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Vol. XXXIII. *October, 1917.* *No. 4*

BIOLOGICAL BULLETIN

INHERITABLE MODIFICATION OF THE WATER RELATION IN HIBERNATION OF LEPTINOTARSA DECEM-LINEATA.

WILLIAM LAWRENCE TOWER.

A series of experiments in which *Leptinotarsa decem-lineata* was introduced into the environmental complexes of the deserts at Tucson, Ariz., in nine years of experimentation, have given the results described in this paper; and show how the introduction of an organism from one habitat into another—in these experiments from a mesophytic into desert environment—produce alteration of the water relation in ways that are adaptive in direction and inheritable in character.

The specific result of the experiments concerns the development in the organism, during the period of the experiments, of the capacity to hold water within the tissues so that the intense desiccation of the dry seasons—which are passed in hibernation—does not result in death, and the elimination of the introduced population. The change is adaptive; directly in line with the environmental pressure that is incident upon the population, and in tests is shown to be gametic; in crossing with the normal behaving as a Mendelian dominant; and is not easily reversible.

The materials in the entire series have been pure strains from my laboratory at Chicago; their history, reactions, and genetic composition known for generations before introduction into the Arizona deserts. The materials have at no period in their history been subjected to any experimental operations at Chicago, but are the natural animals, reared under normal conditions, under continuous observation and record. Two strains have been used, No. 99 and No. 100, both derived from nature on the

banks of the Calumet River near Chicago, and therefore characteristic of, and in their activities, adjusted to the conditions of the place of origin.

These stocks have two yearly generations: pass the winter in the adult condition, hibernate in the soil, emerge in the spring, as soon as the temperature is high enough; and begin breeding to produce the first summer generation which matures in July; this first summer generation breeds at once, giving the second summer generation maturing in August or early September. These do not breed, but after feeding undergo preparations for, and enter, hibernation during September or early October, where they remain until the arrival of awakening conditions in the following spring.

In nature this reproductive rhythm is never pure nor regular; many of the overwintering population remain alive, or are late in emergence from hibernation, and are able, therefore, to cross with the first summer generation, giving hybrids between two differing conditions in the rhythm of reproduction, and all materials direct from nature are liable to be in one way or another heterozygous with respect to this rhythm. Pure lines, or stocks in which the rhythm of reproduction was homozygous, have been used in these experiments and no others.

The second summer generation does not mature its gametes, and after feeding undergoes changes preparatory to hibernation that are common to insects; the elimination of waste products and the reduction of the water content of the tissues, lowering the freezing point thereof, so that the animals are not killed by the low temperatures of the winter months, an operation common to many plants and animals in resting conditions. Upon emergence in the spring, or for breeding, this water reduction is rapidly compensated for by water derived from the food and that absorbed by the tissues from the atmopshere or hygroscopic water.

The location chosen for the experiments at the Desert Laboratory of the Carnegie Institution of Washington was on the edge of the broad flood plain of the Santa Cruz River at the foot of Tumamoc Hill, a deposit of gravel and boulder beds with a thick surface layer of adobe soil, of uniform texture and com-

position. All experiments were in cages six feet square and three feet high, with wooden or cement bases that extended into the ground two and one half feet, or below the depth to which the animals burrowed in hibernation. These cages were covered with sixteen mesh pearl wire cloth, which acted to soften the rigors of the desert environment, especially the temperature, rate of evaporation and wind flow, so that the materials while subjected to the rigors of the desert did not receive the whole intensity thereof, but approximately the average conditions.

Throughout the food has been the cultivated potato, as in the original habitat of the materials. All watering was by irrigation about the roots of the plants, and in all respects care was taken to prevent the experiments from having any other than the desert environment about them.

Caged experiments were used instead of isolated locations in the open for two reasons: first, observation and record, as well as protection, could best be maintained in the location and in the arrangements as devised; and second, in caged experiments all selective action by animals that might use these beetles as food was eliminated and the results would, as fully as possible, be those directly due to the physical environmental complex. It developed in the course of the experiments that the arrangements provided not only eliminated this source of complication, but that predaceous enemies, parasites, and epidemics were also absent, no trace of any of them having been discovered, so that as far as I am able to discover, the results are solely the product of the action of the physical environment upon the introduced animals.

The Experiments.

All materials were introduced at the opening of the summer rains, and taken directly from hibernation to Tucson and introduced into the cages, there giving two generations in rapid succession, the second going into hibernation late in the summer, depending upon the environic condition. The following series have given the data for this paper:

T 99, introduced 1908, now in hibernation F_{13}.

T 100, introduced 1909, carried to F_{12} and lost in making repairs.

T 100 A, introduced 1911, now in hibernation in F_{12}.

T 100 B, introduced 1912, now in hibernation in F_{10}.
T 100 C, introduced 1915, now in hibernation in F_4.

Each introduction was taken from the stock at Chicago in the following way: On emergence from hibernation the materials were, of each sex, placed in containers, covered with a cloth, and twenty to twenty-five of each sex removed without inspection, so that there would be no opportunity for selective action on my part, and this parental group of forty to fifty individuals were taken or sent at once to Tucson and placed in the cage prepared with food to receive them. They were then allowed to breed as a population. When the progeny of an introduced group, or if at any other time the population was so large that to breed all was impossible, the same impersonal method of deriving the parental group was resorted to, and it seems highly improbable that with these methods, any selective action could have entered into the series of experiments.

Each introduction has had the same method of derivation from the basal stocks at Chicago, and the same treatment under the conditions of experiment at Tucson, grown as populations, treated as populations, and the results are of the population as a whole, and not of individual lines or mutants.

The series has shown several interesting responses to the introduction into the desert environment, but the most interesting one is the development of a capacity to retain water in the tissues of the second summer, or hibernating generation, instead of eliminating it in preparation for hibernation.

The development of this modification of the animal was first discovered in F_6 of T 99, when a population of over four hundred individuals of the second annual generation was sent to Chicago early in September, 1910 (211 ♂ 234 ♀), placed in a cage in the garden at Chicago, in size and construction like those at Tucson, where they soon entered into hibernation, but absolutely failed to survive the Chicago winter, and were completely eliminated, while a culture of the parent stock in a cage six feet away showed only the normal reaction to that particular winter.

The following year, 1911, tests of this condition were made as follows: T 99 in F_8, of the second annual generation, and T 100 in F_2 of the second annual generation were sent to Chicago

early in September and hibernated in the garden alongside of the original stocks, and a further test was made of T 99 in F_7 in which the first summer generation was crossed as pairs with the normal stock at Chicago, and their F_1 allowed to hibernate in the garden along with the normal and the stocks from Tucson. The results that were derived in the following spring are shown in Table I.

TABLE I.

SHOWING THE RESULTS OF THE TESTING OF THE SURVIVAL DURING HIBERNATION IN THE WINTER AT CHICAGO.

Population Generation, Stock.	Test, Winter of	No.	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
T 99, F_3.....	1911–12	100♂	87♂	0♂	13	87
		100♀	90♀	0♀	10	90
T 100, F_2	1911–12	100♂	86♂	5♂	14	80
		100♀	89♀	7♀	11	82
*C 99, F_{20}.....	1911–12	100♂	91♂	80♂	9	11
		100♀	89♀	84♀	11	16
C 100, F_{12}.....	1911–12	100♂	92♂	83♂	8	17
		100♀	87♀	79♀	13	8
F_1 Heterozygotes:						
♂ T 99 × ♀ C 99.....		347♂	317♂	0♂	8.6	91.4
♀ T 99 × ♂ C 99.....		386♀	342♀	0♀	11.4	88.6

* Chicago normal stock.

These tests were made in wire tubes twelve inches in diameter and forty-eight inches long, placed close together, so that all had as far as one can determine the same conditions during the winter. The findings in the emergence from hibernation in May and June, 1912, show, first, that the condition of non-survival in the T 99 materials concerns the entire population, and that in T 100, the same change is coming over the stock but did not involve the entire population, 6.5 per cent. surviving. These surviving individuals of T 100 were placed in breeding cages, where most of them died, two females and one male only taking part in reproduction, giving a first summer generation of eleven males and fifteen females, which died without breeding, although given every condition for reproduction.

The F_1 heterozygous population showed complete elimination, or a total dominance of the condition present in the Tucson parent, which was also present in an equal intensity in both

parents, and equally dominant. On the contrary, the Chicago stocks showed a rather good rate of survival, about the average, and in that the entire test was in a space not over six feet square, with a uniform sandy soil, the result can hardly be attributed to highly localized differences in the winter conditions.

In the summer of 1912 further and more extensive tests were made as follows:

T 99 in F_{10} in the second summer generation to Chicago in September.

T 100 in F_4 in the second summer generation to Chicago in September.

T 100 A in F_2 in the second summer generation to Chicago in September.

The tests for the year comprise the determination of survival in populations that had been at Tucson two, four and ten generations. The results of these tests as determined in the spring of 1913 are given in Table II.

TABLE II.

Population Generation, Stock.	Test, Winter of	No.	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
T 99, F_{10}.....	1912–13	100♂	89♂	0♂	11	89
		100♀	92♀	0♀	8	92
T 100, F_4.....	1912–13	100♂	87♂	1♂	13	86
		100♀	86♀	3♀	14	83
T 100 A, F_2....	1912–13	100♂	91♂	6♂	9	85
		100♀	87♀	7♀	13	80

In T 99 no survivals were found, and the survivals from T 100, although given opportunity to breed, died without issue, and the survivors from T 100 A gave a first summer generation of twenty-six males and thirty-seven females, these a second summer generation of fifty-five males and forty-seven females, of which two males and three females were able to survive hibernation in the following winter, but did not breed after their emergence from hibernation in the spring of 1913.

In the F_1 hybrids in this year (1912), regardless of the direction of the cross, all were eliminated in hibernation, not one appearing in the following spring, again showing the complete dominance of the Tucson trait over the original or normal condition at

Chicago. In the F_2, however, in both crosses survivals occur, and out of 2,137 hibernated, 443 emerged in the spring of 1913. Two random matings were made from these, of twenty males and twenty females, and large populations grown for the second summer generation and hibernated under the usual conditions, gave in the spring of 1914 the following results:

	Hibernated.		Emerged.	
	Males.	Females.	Males.	Females.
Mating *A*	861	905	672	689
Mating *B*	919	945	792	807

These in all ways were not to be distinguished from the normal stocks, and allowing for the normal elimination incident to hibernation under the conditions provided, which averages in my garden about twenty per cent., the experiences of the experiment suggest that we have in the F_2 popuulation of 1912

	Completely Eliminated. 1 DD. + 2 Dr.	Surviving. 1 rr.	
Observed	1,694	443	
Expected	1,602.75	534.25	if a normal monohybrid.

In that the normal elimination in hibernation of the Chicago stock runs about twenty per cent., the eliminated individuals in this test of F_2 are surely the combined DD and Dr portions of the population, plus the non-distinguishable dead individuals of the (rr) portion of the array.

This method of experimentation and testing has been continued in each year to the date of writing, the results of which show that the condition of non-capacity to survive the winter at Chicago is a gradually increasing product of the populations at Tucson, and that the behavior in crossing of the Tucson characteristic is uniformly that of a Mendelian dominant, the normal Chicago condition appearing in F_2 in numbers that are as close to expectation as could be expected.

Since the discovery of this condition in T 99, all of the introductions of *L. decem-lineata* have been tested in the hibernating generation with respect to their capacity to survive the winter conditions of the habitat from which the stock came. The results of these tests are presented in Tables III., IV., V. and VI.

TABLE III.

SHOWING IN CULTURE T 100, THE COMPLETE FAILURE OF SURVIVAL IN THE POPULATIONS OF THIS CULTURE AFTER SIX GENERATIONS IN THE DESERTS OF TUCSON.

Population Generation.	Test Winter of	Number Taken.	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
F_6	1911–12	100♂	87♂	0♂	13	87
		100♀	90♀	0♀	10	90
F_8	1912–13	50♂	47♂	0♂	6	94
		50♀	44♀	0♀	12	88
F_{10}	1913–14	25♂	23♂	0♂	8	92
		25♀	22♀	0♀	12	88
F_{12}	1914–15	50♂	44♂	0♂	12	88
		40♀	39♀	0♀	22	78

TABLE IV.

SHOWING THE RESULTS OF THE TESTS IN CULTURE T 100 A, IN WHICH ELIMINATION DURING HIBERNATION IS SHOWN TO INCREASE PROGRESSIVELY, ENDING IN A CONDITION IN WHICH SURVIVAL OF THE WINTER AT CHICAGO IS NO LONGER POSSIBLE FOR THE POPULATIONS IN THIS CULTURE.

Population Generation.	Test Winter of	Number Taken.	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
F_2	1911–12	100♂	91♀	6♂	9	85
		100♀	87♀	7♀	13	80
F_4	1912–13	100♂	84♂	1♂	16	83
		100♀	87♀	3♀	13	84
F_6	1913–14	50♂	44♂	0♂	12	88
		50♀	45♀	0♀	10	90
F_8	1914–15	100♂	89♂	0♂	11	89
		100♀	93♀	0♀	7	93

TABLE V.

SHOWING THE SURVIVAL TESTS MADE IN THE SECOND, FOURTH, SIXTH AND EIGHTH GENERATIONS OF THE CULTURE T 100 B, IN WHICH THE PROGRESSIVE INCREASE IN THE ELIMINATION DURING HIBERNATION FROM THE SECOND TO THE EIGHTH GENERATION, RESULTS IN COMPLETE ELIMINATION IN THE LAST TWO TESTS MADE IN THIS SERIES.

Population Generation.	Test Winter of	Number Taken.	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
F_2	1912–13	100♂	89♂	5♂	11	84
		100♀	86♀	7♀	14	79
F_4	1913–14	100♂	91♂	2♂	9	89
		100♀	90♀	3♀	10	87
F_6	1914–15	100♂	86♂	0♂	14	86
		100♀	87♀	1♀ died	13	86
F_8	1915–16	50♂	45♂	0♂	10	90
		50♀	42♀	0♀	16	84

TABLE VI.

SHOWING THE TEST MADE UPON CULTURE T 100 C, INTRODUCED INTO THE TUCSON DESERTS IN JUNE, 1915, WHERE IT REPRODUCED, THE SAMPLE OF THE SECOND SUMMER GENERATION, TAKEN TO CHICAGO, IN SEPTEMBER, AND SUBJECTED TO THE SURVIVAL TEST IN THE WINTER OF 1915–16.

Population Generation.	Test Winter of	Number Taken.	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
F_1.....	1915–16	50♂	46♂	4♂	8	84
		59♀	44♀	6♀	12	76

These tests shown in the above tables have been made in the wire tubes, placed close together, and having as far as could be determined the same conditions during a given winter. Undoubtedly the testing in tubes favors the culture in the elimination of possible enemies in the soil, and in fact, the survival of the normal stocks in the tubes at Chicago is higher than in large open cages, so that any action of the tube is favorable to survival rather than otherwise.

In all of the introductions precisely the same action is going on, and in all of the tests the same result shows at about the same rate of progression, namely, that the capacity to survive the winter diminishes progressively as the number of generations increases in which the stocks have lived under the conditions of the Arizona deserts. The greatest change comes between introduction and the hibernation of the first year.

At Chicago for some years I have made routine tests of certain of the activities concerned in hibernation, in that this period of passing of the unfavorable portion of the year in their habitat is a season of possible elimination, of possible selective action, therefore, one of possible effectiveness in the evolution of the population. In Table VII. I have shown a series of tests made in the stock C 100 from the eighth to the eighteenth generations, inclusive. These were all made in wire tubes sunk in the ground, under similar conditions of soil, moisture and climatic exposure.

The tests in Table VII. show that in the preparation for hibernation, on the average, eleven per cent., of the tested materials are not able to complete the preparations and do not enter into hibernation. These will be found dead upon the surface of

TABLE VII.

SHOWING IN THE ORIGINAL CULTURE OF *L. decem-lineata* NO. 100, AT CHICAGO. TESTS MADE TO DETERMINE THE ELIMINATION IN THE HIBERNATING POPULATION, IN PREPARATION FOR HIBERNATION, THE NUMBER THAT HIBERNATED, AND THE PERCENTAGE ELIMINATED DURING HIBERNATION, WITH THE PERCENTAGE OF SURVIVALS IN THE FOLLOWING SPRING.

Stock Culture No. 100, Chicago.

Population Generation.	Test Winter of	Number Taken	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
F_9	1910–11	100♂	91♂	83♂	9	8
		100♀	87♀	80♀	13	7
F_{10}	1911–12	100♂	96♂	82♂	4	14
		100♀	83♀	79♀	17	4
F_{11}	1912–13	100♂	94♂	80♂	6	14
		100♀	88♀	77♀	12	11
F_{14}	1913–14	200♂	181♂	160♂	9.5	10.5
		200♀	173♀	164♀	13.5	5
F_{15}	1914–15	100♂	88♂	71♂	12	7
		100♀	90♀	67♀	10	13
F_{16}	1915–16	100♂	83♂	77♂	17	6
		100♀	91♀	74♀	9	15
Totals		700♂	633♂	553♂	av. 9.583+	av. 9.916+
		700♀	612♀	541♀	av. 12.416+	av. 9.166+
		1,400	1,245	1,094	av. 11	av. 9.541+
Total elimination in connection with hibernation, av.					20.54+	

the ground in the tube, but what the cause of the elimination is has not been determined, and may possibly not be possible of determination as the population will not breed until after hibernation and it is therefore impossible to attempt to get a culture of these individuals for further testing, and further there are no indications that have been detected that they are in any way different from the remainder of the tested population. Moreover the sample for testing has always been drawn from the stock culture, in an absolutely impersonal manner and at random so that the elimination may for the present be regarded as a chance elimination in the preparations for hibernation, of about the same value in each hibernating generation, therefore of no probable modifying action upon the population.

From the same table it appears that during hibernation there is on the average an elimination of nine and one half per cent.

of the tested populations, or a total elimination incident to hibernation of the second summer generation of these animals of about twenty per cent. of the population, about eighty per cent. emerging in the following spring. In nature, however, the elimination is greater, due no doubt to agencies that are eliminated under the conditions of these tests, which completely cut off all the losses that might come from burrowing animals that might use them as food, and probably other agencies as well.

In Table VIII. are given the combined results of the testing of the introduced cultures at Tucson, respecting their ability to survive the hibernating season of their native habitat.

TABLE VIII.

SHOWING THE COMBINED RESULTS OF THE TESTING OF THE CULTURES T 99, T 100, T 100 A, T 100 B, T 100 C, FROM THE SECOND TO AND INCLUSIVE OF THE SIXTEENTH GENERATIONS. IT SHOULD BE NOTED THAT THE PERCENTAGE OF ELIMINATION BEFORE HIBERNATION, REMAINS ABOUT AT THE AVERAGE FOR THE SPECIES AS SHOWN IN TABLE BUT THAT THE ELIMINATION DURING HIBERNATION INCREASES, UNTIL ABOUT THE SIXTH GENERATION, WHEN IT BECOMES TOTAL, AND REMAINS SO TO THE END OF THE SERIES TO DATE.

	Population.	No.	Hibernated.	Emerged.	Per Cent. Eliminated.		Per Cent. of Population Surviving.
					Before Hib.	During Hib.	
F_2	T 100 A....	250♂	226♂	15♂	9.33+	84.33+	7.5
	T 100 B....	250♀	217♀	20♀	12.66+	78.66+	
	T 100 C....						
F_4	T 1100 A....	200♂	175♂	3♂	12.5	86	2.25
	T 100 B....	200♀	177♀	6♀	11.5	85.5	
F_6	T 100.....	250♂	217♂	9♂	13	87	.20
	T 100 A....	250♀	222♀	1♀ died	11	88.66+	
	T 100 B....						
F_8	T 99.....	300♂	268♂	0♂	10	90	0
	T 100.....	300♀	270♀	0♀	11	89	
	T 100 A....						
	T 100 B....						
F_{10}	T 99.....	125♂	112♂	0♂	9.5	90.5	0
	T 100.....	125♀	114♀	0♀	10	90	
F_{12}	T 99.....	200♂	177♂	0♂	12	88	0
	T 100.....	200♀	178♀	0♀	11	89	
F_{14}	T 99.....	100♂	89♂	0♂	11	89	0
		100♀	93♀	0♀	7	93	
F_{16}	T 99.....	100♂	86♂	0♂	14	86	0
		100♀	90♀	0♀	10	90	

Comparison of the data in Tables VII. and VIII. shows conclusively: First, that the elimination in the preparation for

hibernation remains at about a constant figure in both the introduced materials at Tucson and in the original materials that have lived continuously at Chicago; second, that the elimination during hibernation takes a great increase in all the second generations tested, from 9.5 per cent. to over 80 per cent., and then in the next following years increases slowly until the sixth, when elimination is complete and continues to be so, no capacity to resist the Chicago winter conditions appearing in any of the Tucson materials in the later generations.

Beyond question, therefore, some change has taken place as a consequence of the introduction of *L. decem-lineata* from the

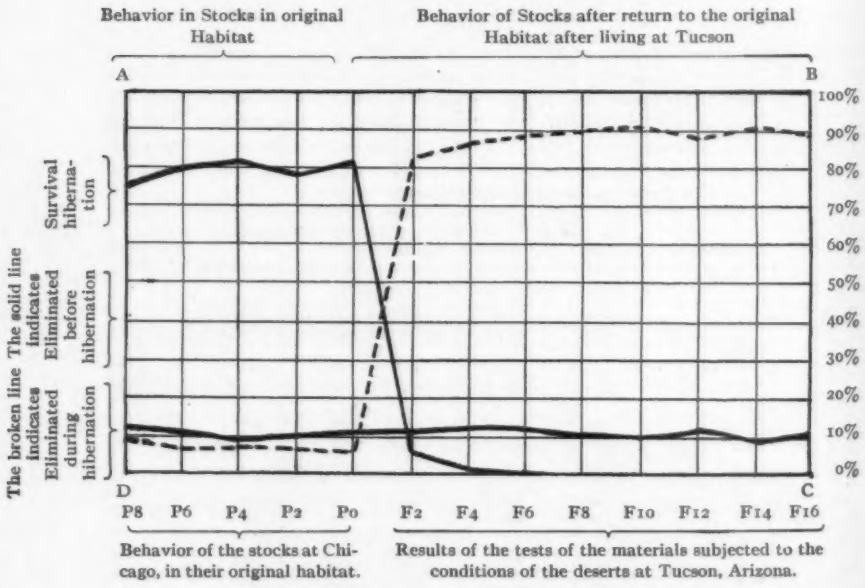

FIG. 1. Graph showing the curve of survivals from hibernation, of elimination in preparation for hibernation, and of elimination during hibernation.

habitat at Chicago into the deserts at Tucson, in the change from a moist habitat into one of intense desiccation during the resting season, which is passed in hibernation. This change is made graphic in Fig. 1, showing the line of elimination in the preparation for hibernation, remaining constant throughout the

series, the sharp changes in the line of survival of hibernation and in elimination during hibernation that occur between the introduction and the first hibernating period, at the end of the second generation.

In hibernation temperature and humidity in the soil are the two chief factors concerned with these animals, and their action upon the organisms is difficult of isolation. In the two environments chosen for the experiments, the conspicuous difference is one of humidity, not of temperature; soil conditions at Chicago during the period of hibernation having a high and rather constant water content, whereas at Tucson the relation is one of continuously diminishing humidity. At the close of the summer rains at Tucson, the intense desiccation accompanying the high temperatures of the dry after summer, rapidly lowers the water content in the soils until the onset of the winter rains in December, and again in the dry fore summer from the end of the winter rains in March to the beginning of the summer rains in July, the water content is progressively lowered until at the end of the period it may be as low as 5 per cent. or even less in the upper layers of the soil. It is this latter period in which the most intensive elimination of the cultures at Tucson takes place.

In desert organisms, in general, some mechanism is present by which the water content of the resting organisms is conserved during the dry and resting periods in the year, otherwise the plants or animals are not able to survive the hostile conditions of these rigorous portions of the year that are so characteristic of every desert environmental complex, and which may be prolonged, irregular, and highly variable in their manifestation in different years. Consequently, surviving desert types must meet, not only the average extreme and hostile conditions in their existence, but irregular and extreme manifestations of the environic complexes, often of prolonged duration. In other words, a desert environment is not only a most rigorous one, but also about the most variable habitat that an organism has to meet.

The response found in these experiments at Tucson suggests at once that the alteration is one of water relations, one in

which the capacity to hold the water in the hibernating generation has developed. Tests of the capacity to hold the water in the tissues against desiccation have been made in the different series of experiments, with rather striking results.

These tests are made by taking about equal weights of one of the Tucson cultures from the generation that is hibernating, and from the cultures at Chicago, enclosing them in small

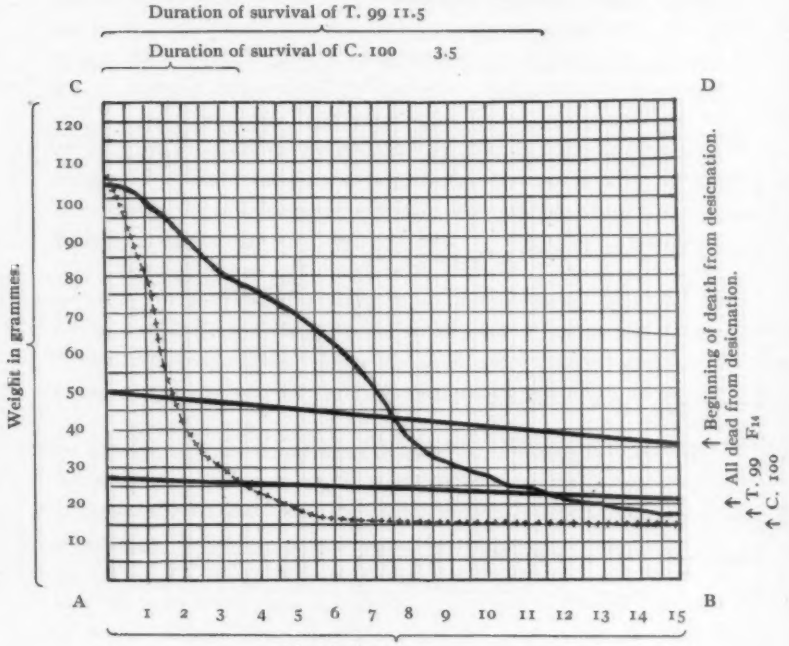

FIG. 2.

wire cloth containers, the two, then subjected to intense desiccation by the passage of a current of dehydrated air at a uniform rate through the materials until death has resulted, and the materials attained a uniform dry weight, weighings being taken every twelve hours.

These tests show that the Tucson materials, especially after

the sixth generation at Tucson, have constantly the capacity to hold onto the water content in their tissues, are more resistant to desiccation, and in every way are different in their reactions to the water loss than are the same materials that have lived at Chicago continuously. In Fig. 2 I have plotted the results of one of these dual tests made upon T 99 in the fourteenth generation of its existence at Tucson, and the stock C 100 from Chicago.

The curve of response of the materials at Chicago shows a rapid water loss in the first day, the onset of death from desiccation by the end of the second, and the death of all in this test at three and one half days, with the attainment of a constant dry weight under the conditions at about the sixth day. In the sample from Tucson, however, the curve of response to desiccation shows a much less rapid loss, the first deaths not occurring until the seventh day and total death not until the twelfth day, and dry weight constancy about the fifteenth, but not as low as in the culture of Chicago materials. The temperature in this test was constantly 20° C., the temperature used in all such testings.

There is a very obvious difference in the ability of the two sets of organisms to let go of the water in the tissues under-desiccating conditions in the environment. In these tests the conditions were most severe, with absolutely dry and rapid passage of air, in all respects immensely more severe than are ever obtained in any desert complex during hibernation, as the animals are then protected in the ground and desiccation is slow and gradual. However, the desert adjusted group shows that it has the ability to hold the water, or less capacity to let the water loose from its tissues when subjected to the pull of an intensely dry environic condition, and this one difference would decide the fate of either set of materials in different environments.

The eliminations in the survival tests made at Chicago of the samples from the Tucson stocks, are clearly due to their not being able to let go of the water in their tissues with sufficient rapidity to keep the freezing point below the temperature of the soil, consequently they are actually frozen by the decreasing temperatures accompanying the onset of the northern winter, and examination of test cultures made for the purpose at Chicago shows

that few of the individuals in the test survive beyond December first, and none have been found alive by the end of the same month. Further many of the individuals seen in these examinations when recovered from the ground show frost crystals within the tissues, and extensive disruptive actions thereof.

Repeated tests in the different cultures at Tucson, at early and late generations during their history, show that the retention of water in the tissues against the influences of a strongly desiccating environic complex gives a series of findings that are in close accord with the data derived from the survival tests of the same materials at Chicago; a sharp change in this capacity between introduction and the first period of hibernation, and thereafter a slower increase through the following hibernation periods, until the full capacity is developed, at about the sixth generation after introduction.

From all the different aspects in which this response to the changed environic complex is viewed, it gives only the one conclusion, that there has resulted from the sharp change in the living conditions the development of a capacity to hold water and not lose water in the presence of conditions in the surroundings, which in the original materials causes rapid loss of water with eventual death. The modification is, therefore, of a direct adaptive nature, in direction and kind to meet the needs of the new environment.

What the alteration has actually been in the mechanism of the organism, I do not know. Thus far I have not been able to detect any anatomical modifications, or changes of a cytological character that have taken place. Water loss in these animals is through the dermal glandular secretions and respiratory activity. As far as I can determine, there is no decrease in the dermal glands that are present, either in size or number, or any alteration in their distribution, although I have searched diligently therefor. Moreover, there are no indications of a thicker cell wall, either in the hypodermis or of any increased thickness in the cuticular linings of the tracheal tubes.

Whether it is due to changes in the permeability of the cell membranes or to changes in the colloidal contents of the cells is a matter of opinion, as I have no evidence that supports

either view with any certainty, and accurate determination of either of these is difficult, if not quite impossible. Regardless of what the actual change may consist of, it is an evolutionary modification, that is directly adaptive in response to altered conditions of life, and as such is well worth careful examination and further investigation.

Perchance it may seem that it is easy to arrive at results of this kind. To prevent any such misapprehension, the experiences in the introduction of other species into the same environic complexes may be cited. During the years in which these experiments with *L. decem-lineata* have been in progress, there have been introduced with the same care and persistency *L. signaticollis*, *diversa*, *undecimlineata*, *panamensis*, *multitæniata*, *oblongata*, *haldemani*, *juncta*, and *dilecta*, representing a diversity of environmental origins and adjustments to temperature and moisture during the hibernation period, and in none of them has anything like the result described in *decem-lineata* been found. The closest approximations thereto are in the species *multitæniata*, *oblongata*, and *signaticollis*, all of which live in haibtats having rather high rates of desiccation during the resting season, and survival in the case of these introductions has been irregular, and the product of favorable conditions in the particular winter, rather than any alteration resulting from the introduction. All of these species, further, live in areas in which the temperature in the resting season is fairly high, so that there is no necessity for the reduction of the water content to prevent elimination by frost action.

Inheritance.

It was shown in connection with some of the earlier tests that the alteration when crossed with the normal condition at Chicago, gave an F_1 that was completely eliminated during the test of survival of the winter, and that F_2 showed a result suggesting the survival of the recessive extractives of the normal type. Further crossings of these have confirmed this original finding and extended the information as to the behavior of the alteration in inheritance, thus showing that the change between the conditions of the populations at Chicago and Tucson is of a gametic nature, and not somatic or temporary.

All tests of the heritability of this modification have been made with the sixth or later generations, for the reason that the alteration was apparently not at its full development until then, and in that the only means of distinguishing between the two conditions has been the survival test under the winter conditions at Chicago, earlier generations in which there were survivals of this test, would have given only results that had an error in them not possible of estimation. After the sixth generation at Tucson, all the cultures have thus far given the uniform result in crossings with the normal at Chicago, regardless of the direction of the cross, and these summated results are given in Table IX.

TABLE IX.

F_1 Populations.	Pairs.	Population.	Total Tested.	Hibernated.	Emerged.	% Eliminated.		Survived.
						Before.	During.	
Pairs { T. 99 × C. 100 / C. 100 × T. 100 / T. 99 × C. 99 / C. 99 × T. 99 }	42	7	2,987	2,651	0	11.25 ±	88.75 ±	0
F_2 population.....	65	11	6,411	5,814	1,573	9.3 ±	66.1 ±	24.4 ±

In making these tests of the heritability of the alteration in the Tucson cultures, the F_1 series were all from matings of the first summer generation, whose progeny would hibernate, and for the F_2 tests, the over-wintering generations at Tucson and Chicago were crossed at Chicago, and the second summer generation tested. The results show in all that the F_1 population are in no instance able to survive the test, that the condition of the Tucson cultures is dominant, but that there is a segregation in F_2 and a survival from the test that is for the total series 24.4 per cent., about the expected proportion of the extracted recessives. These in breeding in F_3 show no indication of the Tucson condition, either in pairs or mass cultures, all surviving the hibernation test in F_4 in all respects like the normal Chicago materials.

The behavior in inheritance shows, therefore, no points of interest aside from the fact of its heritability, and the fact that a condition that has arisen rapidly, but apparently progressively, behaves in crossing when fully established as a Mendelian

dominant, but its behavior in a Mendelian way is no indication of its origin after its production.

Nature of the Alteration.

As already shown, the alteration in the Tucson cultures is physiological, involving a specific aspect of the reproductive cycle, that of the resting period which is passed in hibernation, and the only sure test of its existence is that of the survival under the conditions of the northern winter, at the place of origin of the stocks. This fact limits the possibilities of testing and experiments with the modifications that are possible, since no certain structural alterations in correlation with this change are demonstrated up to the present, and the differences in the color in the materials are too insecure criteria to be used at present.

What the physical or chemical changes are there is no indication at the present time, and one interpretation has about the same chances of being the correct one as any other excepting for the fact that the modified materials show a difficulty or slowness in water loss which seems to me to suggest more probably some altered condition in the colloids.

The chief question that concerns us in the consideration of the nature of the alteration, is whether it is an added or new condition, or the revival of an ancestral one.

The species *L. decem-lineata*, originally confined to the eastern slopes of the Rocky Mountain uplift, and eastward over the Great Plains into western Kansas and Nebraska, from whence it gradually spread eastward to the Atlantic coast, has as its nearest relatives, a series of species that are confined to the high plateaus of Mexico; areas of semi-desert with arid conditions, especially in the resting season. Using the usual criteria of kinship, similarity in structures and habits, the species *L. multitæniata* and *oblongata* are the nearest relations of this northern species phylogenetically.

All three species interbreed rather freely, are in developmental sequences similar, as in their ecological relations, in their reproductive cycles, in relation to the growing season of the habitat in which they live. These relations and conditions I have

recorded in a previous publication, along with the probable relations and phyletic history and relations of these organisms.

Both of the southern species in all of the locations in which I have information concerning them, must encounter during their period of hibernation desiccation in differing degrees of intensity, from a rather low one on the Plateau of Ahnuac, to intense conditions in the deserts of San Luis Potosi, and in hibernation do not go deep in the soils, but remain in the upper layers, or near the surface, whereas *decem-lineata* in its range goes deep in the soil. Owing to the disturbed and impossible conditions in Mexico, since this condition was discovered in the Tucson cultures, it has been impossible to either obtain new materials from the Mexican locations, or make desirable observations and tests that might have been made had more happy conditions prevailed. Fortunately some experiments made in 1908 and 1909 for other purposes have given data as a by-product that is of interest in this connection.

In both of these years crosses were made in the garden at Chicago of the C 100 stock and freshly obtained materials of both *L. oblongata* and *L. multitæniata*, the latter from Chapultepec near the City of Mexico, the former from Cuernavaca in the State of Morelos, Mexico. These were of the first summer generation in all species, and the F_1 populations were hibernated in the garden at Chicago under the full intensity of the winters of 1908–9 and 1909–10, with the results that are shown in Table X., all the materials being from the mating of pairs, and not from population cultures.

The experiences in the crossing of the three species shown in Table X. with the elimination of the hibernation F_1 population during the winter months is highly suggestive, and would give the basis for an interesting series of tests were it possible to obtain fresh materials from the Mexican locations. The complete dominance of the Mexican species over *decem-lineata* in the matter of survival in hibernation, indicates that in them the same or essentially the same condition exists as in the cultures at Tucson. Unfortunately, I have not had the necessary materials for the testing of their resistance to desiccation, and can therefore only conclude that the close identity in the survivals

from hibernation is due to the same causes as it is in the tests of the Tucson cultures. Unfortunately, I have no data concerning the survival of the Mexican types at Chicago that are at all comparable, so that I am not sure whether the pure Mexican

TABLE X.

		Pairs.	F_1.		Hibernated.		Emerged.	
L. 10 lin. C. 100 ♂×♀ L. mult Chap.*	1908–09	7	170♂	196♀	147♂	171♀	0♂	1♀ died
	1909–10	14	278♂	304♀	229♂	258♀	2♂ died	1♀ died
" ♀×♂ "	1908–09	6	145♂	144♀	119♂	123♀	0♂	0♀
	1909–10	10	309♂	301♀	267♂	271♀	1♂ died	0♀
			902♂	945♀	762♂	823♀	3♂ died	2♀ died
L. 10 lin. C. 100 ♂×♀ L. obl. Cuer.†	1908–9	4	96♂	104♀	74♂	91♀	0♂	0♀
	1909–10	6	201♂	197♀	161♂	174♀	0♂	0♀
" ♀×♂ "	1908–9	5	131♂	127♀	100♂	93♀	0♂	0♀
	1909–10	9	246♂	265♀	197♂	213♀	0♂	0♀
			674♂	693♀	532♂	571♀	0♂	0♀
L. obl. Cuer. ♂×♀ L. mult. Chap.	1908–9	2	77♂	79♀	64♂	60♀	0♂	0♀
	1909–10	7	193♂	201♀	154♂	183♀	0♂	0♀
" ♀×♂ "	1908–9	3	66♂	73♀	60♂	64♀	0♂	0♀
	1909–10	4	107♂	119♀	91♂	99♀	0♂	0♀
			443♂	472♀	369♂	406♀	0♂	0♀

* Chapultepec. † Cuernavaca.

types are able to survive the Chicago winter or not, but it is my opinion that they would not.

The two Mexican species introduced into the deserts at Tucson showed in the first introductions in 1908 a huge elimination during hibernation, but they survived in the following year and continued a precarious existence for several years thereafter. Later introductions survived, or failed, for reasons not associated with the behavior in hibernation.

Upon the basis of the probable phylogenetic relations, the facts of the developmental cycles and the similarity in the ecological relations and needs, the data in the crossing of the species under the Chicago conditions and the survival values of the F_1 found therein, and the obvious recessiveness of the northern species in its hibernation behavior to the southern, gives the basis for several conclusions of interest as to the nature of the change.

(1) Assuming that the phylogenetic picture that we have drawn of the three species is essentially correct, and that *L. decem-lineata* is the younger derivative from the Mexican types, an essentially fair conclusion as far as conclusions in this line are at all possible, then the results of the tests show that there is a dominance of the older phylogenetic condition over the newer; or

(2) It may be concluded that in the origination of *L. decem-lineata* in its northern range, there had been a loss of an agent in the organization, which when present, produced retention and slow loss of water under desiccation, and its absence ease of loss, the experimental tests showing, therefore, the dominance of the presence over the absence. In that the reaction in crossing is a monohybrid one, one unit of difference may be assumed; or

(3) It may be concluded that we are dealing with a physical state in the living mechanism that exists as two conditions, alternative to one another in crossing reactions, but, which by the pressure of the medium may be altered to present first one aspect of its reaction, and under other conditions of the medium, the opposite aspect of its effect in the reactions of the living mechanism.

Which of the three possibilities is chosen as the correct one will depend entirely upon one's philosophical background, and from each a rather entertaining and plausible argument can be made. From purely mechanistic and physico-chemical considerations of living phenomena, I am inclined to the third possibility, as not only the more probable and thinkable, but also the one that as a working hypothesis offers the only opening for further experimental investigation.

In this preference I am no doubt prejudiced in my choice by many experiences with the behavior of the non-hibernating generation in which the behavior is easily, rapidly and positively altered, reversed at will by changes in the water content of the medium, producing reversibility of many of the trophisms of that generation. However, in this first summer generation, another condition of the reproductive cycle is under observation, and in that the reproductive cycle itself is present in two sharply alternative conditions, to breed or not to breed, activity or

hibernation, and further since these states are known in experiments to be alternative in hereditary behavior, one must not be too fast nor certain in arriving at conclusions in these complicated physiological activities.

Regardless of what one may believe concerning the alteration or the findings of subsequent investigations, certain aspects of its nature are demonstrated, its sharp alternativeness in heredity, its rôle in the determination of survival in different habitats, its directly adaptive nature; and its alteration in direct response to the conditions of the medium in adaptation to those conditions; these aspects of its nature show an alteration, a character capable of alteration, in ways that are of importance in the survival of the organisms, and that might be of decided value in evolutionary activities, speciation, and in ecological and distributional relations and results.

Method of Origin.

This account deals with experiments that are concerned with populations, and the results seem to be those of the alteration of the population as a whole. As shown, the experimental method was such that as far as the operator was aware, the choice of materials was random and entirely impersonal, and throughout any personal influence has been eliminated as far as known. Further, the living of the cultures in cages eliminated the action, if any, of enemies and further gave the cultures better conditions of life than they would have had in nature. Insofar as is known, therefore, the series deals with the response of an introduced population to the physical conditions of its environment. What are the methods of origin of this response and alteration? There are several possibilities:

1. That the original population consists of different pure lines, which under the conditions of the original habitat interbreed, and are hidden from recognition, but that under the Tucson conditions one has been able to survive with the characteristics found, and that the progressive development of its full intensity is the gradual elimination under the conditions of the experiment, of all but the one line with the characteristics finally developed. The conditions of experiment are, therefore, acting only as a sieve to separate out those capable of survival from those not

capable thereof, and we are determining in the experiment only some aspects of the composition of the population of the species in its original habitat.

2. That the introduced populations are uniform in composition, but that as the result of the altered conditions of life, variations are induced, of which only those whose reactions enable them to resist desiccation have survived; a natural selection of the fit by the physical environic factors.

3. That the population has latent within it the factor for water retention, and that this latency from an ancestral conditions under the conditions of the new habitat, is reactivated, mutates to the ancestral state in the materials of the experiments.

4. That the water relation in connection with hibernation is one due to some physico-chemical relation in the living colloids, that exists in one of two aspects, a condition of the substance, its type of manifestation depending upon the nature of the conditions of the medium.

There may be other possible methods whereby the condition is produced in the experiments, but these seem to me the most obvious possibilities.

That the results are due to the isolation under experiment from the introduced population of a pure line does not seem probable, or at all possible under the conditions of these experiments. All the basal populations introduced came from cultures in which the reproductive cycle was known to be homozygous with respect to the rhythm of the two generations, and since any heterozygousness in this rhythm is at once manifested and very obvious in the cultures, and was also repeatedly tested for in the stock cultures, and none found, the evidence is against the assumption. Further all the introduced stocks were taken from the original stocks on emergence from hibernation, after all the elimination incidental to hibernation had taken place. Were there lines in the original materials with the conditions that later developed in the Tucson cultures, which were isolated by the selective action of the Tucson experiments, and could not later survive the Chicago conditions when returned thereto; why, if present in the original stocks, were they not also eliminated by the conditions of the winter hibernation in the parental habitat?

Further in the elimination incident to hibernation, that found in preparation for hibernation remains about a constant percentage (about 11) throughout the series at Chicago and Tucson, the difference in the two is in the elimination that takes place during hibernation. The introduced populations at Tucson were all of individuals that had survived the Chicago winter's hibernation, whereas the materials returned to Tucson for testing did not survive the Chicago winter, as shown in the records of the tests.

There is, therefore, no evidence nor reason to interpret the results of the experiments as being due to the isolation at Tucson of pure lines from the introduced population, and every reason, as shown above, why this interpretation is highly improbable. The pure line concept is so facile and insidious in its possibilities for interpretation, that the result of the series of experiments are easily interpreted to conform therewith.

Since 1910 I have tried to develop pure lines from the materials at Chicago with respect to this water retention, and have not up to the present the least evidence that such exist, nor has the slightest success attended my efforts along that line. I cannot go beyond the facts of experience and must, therefore, conclude that upon the basis of the evidence available, pure lines of the characters developed in the Tucson experiments do not exist in the parental materials, and that the results are not due to the sieve-like action of the Tucson environment upon the introduced population, and I believe that this conclusion is entirely justified upon the basis of facts at present available.

It may be assumed that the condition developed was in correlation with some other characteristic operative at breeding, and that introduction eliminated those not possessed of this trait, but in these introductions, as in the T 99 series, from actual observation the entire introduced population took part in the initial breeding, and nowhere have I found the least indication that there has been any such relation, although I have been upon the lookout for them.

That the results are the product of the selection of favorable or adapted variations surviving from among multifarious variations, regardless of size, a product of natural selection, is without

any confirmation. The chief change takes place between introduction and the first period of hibernation in the elimination during hibernation, and there is therefore no opportunity for the operation of such selective action, even though it is present in the following two or three hibernations. I have tried to devise tests of this point, thus far without any success, and rather extensive breeding of pedigree pairs from these cultures have thus far given only negative results in this line. While I admit its possibility and its value as a basis for argument or interpretation, I am still without any evidences at all that can be used either way concerning this possibility, and am of the opinion that it is not, in view of the findings, a plausible interpretation of the results.

Concerning the possibilities of the reactivation of latent agents, under the conditions of experiment, a plausible interpretation could be made, if the initial assumption is made that such latent agents exist, an assumption that might receive some support from the probable phylogenetic history of these animals. Were any such condition latent in these beetles, it would seem that some indication would have been found of it in the rather extensive experiments that have been in progress in the last ten years, especially those that have to do with the reproductive cycle and hibernation; but as far as I am aware, there has been no indication thereof, and finding of it would have been of considerable value in many of my experiments, and after all the finding of the latency would not in this instance be of much service in the interpretation of the results.

It seems to me that the most plausible interpretation of the results of these experiments is that we are dealing with a case of the response of the entire introduced population, of uniform composition and behavior, in a determinate variation in Darwin's sense. A population reaction to the conditions of a new environment in a characteristic that is capable of existence, and does exist in species now living in alternative conditions depending upon the environic constellation encountered. That the alteration is directly adaptive in its manifestation implies neither purpose nor use, only that in the relation of water in the tissues, mechanistic considerations show at once that only two possible

conditions can exist; to hold the water or to let go of it, and there are no other possibilities. In other words, the alteration is adaptive and directive in its response, because from the physical nature of the materials no other relations are physically possible.

A crucial question is, why has the reaction not been as easily reversible on return to Chicago conditions? In this lies interesting possibilities for investigations now in progress. Up to the present the reversibility of this action has not certainly been obtained, although I have no doubt but that if I can discover the proper relations between the internal mechansim of these beetles and their medium that the reverse alteration of the Tucson condition to that at Chicago will be found.

Whether the reversal ancestralward, or what may be assumed to be an ancestralward direction, is easier than progressively, is for the present is a matter of opinion, and I am unable to discover any physico-chemical reason why it should be so, but we know many reversible actions that go easily through one series of events in one direction, and with more difficulty in the reverse and through another series of operations. In these purely physical and chemical reactions, ancestral influence is not at all involved, and I see no reason to assume the influence of ancestry and phylogenetic relationship in living reactions of the same general physical type.

Evolutionary Significance.

In the evolution of populations, in their habitat adjustments as in their distributional phenomena, reactions, accommodations of this sort may well have played a by no means minor rôle, and the value of adjustments of the kind found in these introduced populations at Tucson in these operations in nature are at once too obvious to need mention. Physiological adaptations of many sorts may well have their origin and owe their diversification to operations of precisely this general type, the fact of their heritability or behavior Mendelianwise having no significance or indication of the method of origin. In fact this and other experiences with these animals through many years strongly inclines me to the conclusion, that Mendelian alternativeness is

no certain criterion that they arose discontinuously. Osborn has presented from the findings of the fossil record, certain facts concerning the phylogeny of characters in mammals that now behave Mendelianwise in crosses, but, which arose, as far as the data of paleontology can decide, by gradual and continuous modification, and not by mutative jumps, and further in that the Mendelian reactions concern only extremes or alternatives in the same quality of the organism, it may result that the entire Mendelian phenomena are but the expressions of characters whose manifestation is capable of easy and reversible manifestation.

The fact that this alteration is inheritable at all, may to some, seem incomprehensible, but it never segregates, its behavior in heredity is always that of a mono-hybrid, and in all experiences it behaves as a property characteristic of the whole, and possibly involves, therefore, an alteration not of determiners in the usual sense, but an alteration of the colloidal matrix of the tissues, soma and germ, which we must regard not as different kinds of substance, but as different expressions of one and the same specific kind of living material.

From this series of experiments, operations that are important in population evolution, especially of introduced populations, are certainly discovered, and has served in my hands for an entering wedge into the experimental investigation of questions concerning the evolution of populations, an aspect of evolution of which there is extremely little experimental analysis or investigation. In nature and the diverse products of the evolution phenomena in nature, our classification, schemes of phylogeny, our experiences are with populations of diverse things, in associated relations, both living and non-living, and more intimate knowledge of, and a broader understanding of these population problems seems most necessary for further progress in evolution investigations.

As an example of the manner, and the change necessary, and its production in the alteration of an organism originally characteristic of a mesophytic habitat into one in all ways adjusted to the rigors of the Arizona deserts, of the nicety of adjustment, its promptness, and direct adaptive nature, these experiments

provide illuminating examples and the basis for continued investigation of the same general aspect of the evolution problems.

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ON THE CAPACITY FOR FERTILIZATION AFTER THE INITIATION OF DEVELOPMENT.[1]

CARL R. MOORE.

I. An Attempt to Fertilize Sea-urchin Eggs Subsequent to Hypertonic Parthenogenesis.

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I. Introduction.

The purpose of the present paper is to present a part of the results of a study of the capacity for fertilization of eggs of the sea-urchin (*Arbacia punctulata*) after the initiation of development by artificial agents. A previous paper[2] by the writer has dealt with some of the phenomena encountered in an attempt to fertilize the eggs of *Arbacia* after a treatment with certain artificial stimuli, *e. g.*, butyric acid and heat. The ideas involved resolve themselves into the question—can eggs that have been activated by artificial parthenogenetic agents, be fertilized by spermatozoa of the same species; or does activation by hypertonic sea-water preclude fertilization as in the case of sperm activation?

[1] From the Marine Biological Laboratory, Wood's Hole, Mass., and the Hull Zoölogical Laboratory, University of Chicago.

[2] Moore, '16.

The egg of the sea-urchin cannot be fertilized before maturation has taken place, even though the spermatozoön may enter the egg. Penetration is not fertilization. However, if a spermatozoön enters immediately after maturation, normal changes characteristic of fertilization are produced. But shortly after the act of fertilization the egg returns again to a condition in which fertilization is impossible. E. B. Wilson[1] has demonstrated for *Cerebratulus* that if pieces of fertilized eggs are dropped into sea-water containing active spermatozoa there is no reaction between the pieces of eggs and sperm; however, with pieces of unfertilized eggs there is a reaction. Delage, too, has noted that starfish eggs which had been exposed to artificial parthenogenetic agents could not be fertilized.[2]

Maturation, then, leads to changes within the egg that make possible initiation of development by either a spermatozoön or an artificial stimulus. And if this initiation of development is brought about, carrying with it of course a change of the egg system, fertilization is thereby rendered impossible.

It would seem strange not to suppose that an egg passes through essentially similar physiological changes during the development from one cell to a swimming pluteus, whether activation has been produced artificially or by a spermatozoön. If activation by a spermatozoön produces a physiological state of reactivity that leads to development, and, if activation by artificial agents also produces physiological changes that lead to the same kind of development, should we not suppose that the egg—the only common factor—would pass through essentially similar conditions in the two cases? One would judge from the papers of Loeb, that this supposition is erroneous, that sperm will react with artificially activated eggs in an entirely normal manner, producing normal swimming larvæ, while it is positively known that such is not the case when activation has been previously affected by means of a spermatozoön.

The writer has presented proof that eggs of the sea-urchin activated by butyric acid, and resulting in the production of normal membranes, will not react with sperm nor become fer-

[1] Wilson, '03.

[2] Delage, '01.

tilized, as Loeb has believed,[1] but that they are entirely unresponsive to sperm, even though spermatozoa pass into the egg substance. The present paper deals with an attempted analysis of the conditions that are produced, after activation by hypertonic sea-water, with reference to the capacity of the egg for fertilization.

Loeb[2] determined that an exposure to hypertonic sea-water (50 c.c. sea-water + 8 c.c. 2½ m. NaCl) for a certain period of time would cause sea-urchin eggs, after their return to normal sea-water, to segment in quite a normal manner: they would also undergo further developmental changes and become essentially normal plutei. But certain eggs from the culture will not have had a sufficiently optimum exposure to bring out their full developmental tendencies and will as a result of this remain quiescent after dividing into two, four or eight cells. Can these eggs activated by hypertonic sea-water yet be fertilized by a spermatozoön? Loeb says they may be and uses the assertion as a proof that the egg has not been changed in its power of normal physiological reactivity; and that it may be fertilized if only a spermatozoön reaches the cortex of the egg. His hypothetical "lysin-like" substance contributed by the spermatozoön can still assert itself by producing cytolysis and his "corrective agent" of the spermatozoön may also act to check the imposed cytolytic condition and allow the egg to go on to development.

If the conditions are as Loeb believes, we will naturally have to assume that activation of the egg—the initiation of development—is accompanied in the two instances: (1) in sperm activation and (2) in activation by artificial parthenogenesis, by a different series of reactions that lead to the same end, namely the production of swimming larvæ. But if this is so we will have to assume a lack of harmony in the development of an egg when activated by different agents. In the vast number of cases in which development may be artificially initiated there is no common factor but the egg itself. Development is a series of reactions on the part of the egg whether initiation is given by a

[1] Loeb, '13.

[2] "Art. Par. and Fert.," pp. 237–238.

spermatozoön or by an artificial agent and, unless we assume such a relatively restricted set of reactions, we have as yet no explanation to offer for parthenogenesis. The principal issue at hand is, can an egg of *Arbacia* that has been once activated by hypertonic sea-water be fertilized or reactivated by a spermatozoön of the same species?

The results obtained from this study show conclusively that a treatment of the sea-urchin egg with hypertonic sea-water for an optimum length of time—*e. g.*, that in which one obtains the highest percentage of development, both in regard to cleavage and to larval production—leads to changes in the physiological state of the egg that result in the loss of its capacity for fertilization. Spermatozoa readily enter the eggs but they are unable to cause a reactivation of the egg; they appear only as inert bodies within the cytoplasm. Not only does the spermatozoön fail to produce activation changes but also the initial changes, produced by the hypertonic treatment, have created an environment in which a spermatozoön very quickly goes to pieces.

If an egg has not received the optimum exposure for activation, from the hypertonic treatment, a spermatozoön is capable to some extent of provoking activation changes, and a very striking gradient of activity on the part of the egg can be demonstrated from the normal behavior in fertilization to a complete state of responsive inactivity. If the previous treatment has led to segmentation, spermatozoa readily enter the blastomeres, but are entirely incapable of reactivating them.

And finally, the capacity for fertilization has been found to correspond with the presence or absence of the egg secretion, fertilizin. If the egg possesses any capacity for fertilization after the hypertonic treatment, fertilizin has been found to be present; when the capacity for fertilization is negative, no fertilizin has ever been detected by its sperm agglutinating properties.

II. Material and Methods.

The material used in this investigation has been confined to the Atlantic sea-urchin (*Arbacia punctulata*) found in the Woods Hole region. Fresh material was received daily from the labor-

atory live-car and was kept in running sea-water in the laboratory until used. Eggs and sperm were secured in the usual way—by cutting away the leathery oral disk and collecting the eggs and sperm in separate syracuse dishes as it is shed by the animal. Eggs were always washed in sea-water from two to four times before being used in an experiment, as this insures a more consistent lot of fertilizations than with unwashed eggs.

The method for testing the capacity for fertilization of eggs that have been activated by a hypertonic solution is the same as that employed by Loeb[1] in his studies of this nature. Eggs after washing were transferred to the hypertonic sea-water and allowed to remain for definite periods of time: at the termination of the exposure they were removed with a pipette to two separate dishes of sea-water (150 c.c.–200 c.c.) A and B. Lot A was allowed to stand at room temperature without further treatment, while to lot B was added a quantity of a fresh sperm suspension. For each experiment both a fertilized and an unfertilized lot were set aside as controls.

III. Observations of Living Material.

Since there is a great difference in the effects of different concentrations of, and variable time exposures to, hypertonic sea-water the experiments with the sea-urchin egg have included quite a range of concentrations and periods of exposure, but to simplify the data, only results from two concentrations of the hypertonic solution will be reported at this time. The two concentrations may then be known as the weaker solution (50 c.c. sea-water + 8 c.c. 2½ m. NaCl) and the stronger solution (50 c.c. sea-water + 16 c.c. 2½ m. NaCl).

These two concentrations of hypertonic sea-water were employed because of the very different results obtained with them. An exposure to the weaker solution results in the segmentation of a relatively few eggs (see Table I. and Fig. 1, *a*) but usually a very small percentage of the eggs will segment once or twice and cease their development, while a similar exposure to the stronger solution gives a much higher percentage of cleavages and a much greater number of swimming larvæ. An exposure

[1] "Art. Par. and Fert.," pp. 235–236.

to the latter solution is much more nearly an optimum treatment for activation than is the weaker concentration.

1. *Fertilization after Exposure to Hypertonic Sea-water.*

The fundamental interest of the writer has been directed towards the capacity of an egg, once activated by an artificial stimulus, for fertilization. If an egg is fertilized normally, the spermatozoön supplies the optimum stimulus for the initiation of changes, latent within it, that lead to normal development. But since these same changes may be initiated (it seems that we must regard it so) by artificial agents, is there yet the possibility of an observable stimulus from a spermatozoön if applied subsequent to or during these changes? Can fertilization be superimposed upon hypertonic parthenogenesis?

Eggs were exposed to the action of hypertonic sea-water as given above, returned to normal sea-water and divided into two lots, one of which remained standing at room temperature, while to the other was added a fresh sperm suspension. Let it again be brought to mind that exceedingly variable results are obtained from the same type of treatment of two different lots of eggs and since the condition of the eggs before sperm are added is the all-important factor for subsequent fertilization one must be very careful to know as thoroughly as possible the condition of the eggs in question. In one experiment one may obtain only 3 per cent. of cleavages after a given hypertonic treatment and at another time, under as nearly as possible identical conditions, 25 per cent. to 35 per cent. cleavages. The following experiment will indicate the method of procedure and the results in general that have been obtained.

From the results of this experiment one can readily see the different capacities for fertilization possessed by eggs exposed to the weaker solution (lot A) and those activated by the stronger one (lot B). This relation can be seen at a glance from Fig. 1, the curves of which were plotted from this experiment.

The lot of eggs returned to normal sea-water and inseminated, after an exposure to the weaker solution A, for a period of ten to twenty minutes, appear very little, if any, different from the control lot of normally inseminated eggs, either as regards

Experiment:

4:30 P.M. Eggs collected and washed. Aug. 26, 1916.

5:00 P.M. Divided into two lots *A* and *B*.

A, placed in (50 c.c. sea-water + 8 c.c. 2½ m. NaCl).

B, placed in (50 c.c. sea-water + 16 c.c. 2½ m. NaCl).

Eggs were returned to sea-water from the two solutions by means of a pipette and divided into two lots one of which remained without further treatment (control): to the other was added immediately a fresh sperm suspension.

TABLE I.

Lot A.

No.	Returned to Sea-water.	Hypertonic Solution Alone. Percentages of		Hypertonic Solution Plus Sperm. Percentages of	
		Cleavages.	Swimming Larvæ.	Cleavages.	Swimming Larvæ.
1	5:10 P.M.	0	0	99	90
2	5:20 "	0	0	95	85
3	5:35 "	1	0	83	70
4	5:50 "	2	0	70	55
5	6:00 "	3	1	60	35
6	6:20 "	2	0	28	20
7	6:35 "	2	2	33	20
8	6:50 "	4	0	30	15
9	7:05 "	5	1	28	5
10	7:20 "	4	1	20	15
11	7:35 "	6	0	35	10
12	7:50 "	8	0	35	5

Lot B.

1	5:05 P.M.	1	0	22	25
2	5:10 "	0	0	18	18
3	5:20 "	10	7	12	20
4	5:30 "	22	20	20	28
5	5:40 "	18	7	18	5
6	5:50 "	14	5	9	2
7	6:00 "	12	1	10	1
8	6:20 "	5	0	1	0
9	6:35 "	6	0	6	0
10	6:50 "	4	0	1	0
11	7:05 "	2	0	2	0

the number of cleavages or the number of swimming larvæ. But since an exposure of this intensity is practically ineffective in producing initiation of development, is it not to be supposed that the eggs can yet be fertilized by sperm? They are, so far as we may judge, entirely normal or essentially so. However, as the length of exposure to the hypertonic solution is prolonged the capacity for fertilization drops off until after an exposure of two hours' duration scarcely any larvæ appear as a result

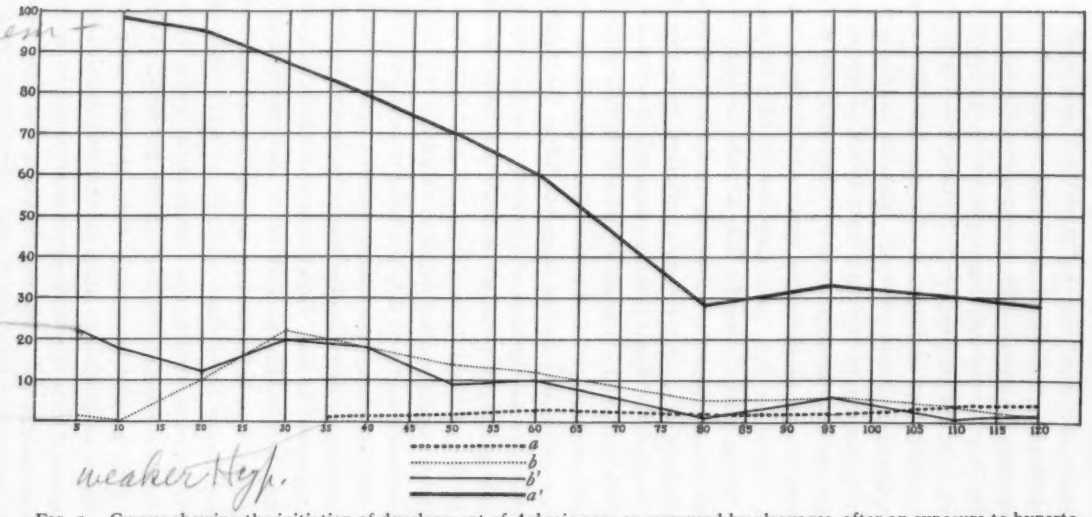

FIG. 1. Curves showing the initiation of development of *Arbacia* eggs as measured by cleavages, after an exposure to hypertonic sea-water; and the relative capacity for fertilization after an exposure to the different concentrations. *a*, weaker hypertonic solution alone; *a'*, weaker hypertonic solution followed by insemination; *b*, stronger hypertonic solution alone; *b'*, stronger hypertonic solution followed by insemination. The ordinates represent percentages of cleavage; and the abscissæ, time of exposure in minutes.

of insemination.[1] This is undoubtedly due to the condition of the egg, produced as a result of the hypertonic exposure· its capacity for fertilization is being gradually decreased.

We may correlate, it seems, this gradually progressing unfertilizable condition of the egg that appears as the eggs are allowed to remain in the solution, and the escape of substances from the eggs which also increases as the eggs are allowed to remain for longer times in the solution. One can readily notice an almost entire absence of pigment from many of these eggs while others will show a decided loss of it. Probably also many other substances escape at the same time from the egg, some of which may be absolutely necessary for certain chemical combinations taking place during fertilization and development.

The type of swimming larvæ obtained in such a series of experiments shows also a very beautiful gradient in normality as well as in number. Most of those obtained as a result of fertilization, after a short hypertonic treatment, appear entirely normal, but the percentage of abnormalities increases as the length of exposure to the hypertonic solution before fertilization was lengthened: after an exposure of 1½ hours to 2 hours practically no normal larvæ are obtained and the mortality is very high.

For the higher concentration the curve (*b* and b^1, Fig. 1) is decidedly different from (*a* and a^1), the weaker one, but yet it is quite characteristic for exposures to such a concentration. The greatest percentage of cleavages from the hypertonic solution alone are obtained in lots, the exposure of which had been thirty minutes or forty minutes in duration while often a small amount of segmentation is induced by an exposure of 1½ hours. But distinctly different from the curve of cleavages when the lower concentration was used is the fact that the curve of fertilization (?) follows more nearly that of the hypertonic treatment alone. This is especially true when the percentage of swimming larvæ is considered. In dishes of inseminated eggs, previously exposed to the stronger hypertonic solution for 10,

[1] The number of cleavages after a two-hour exposure in this experiment is exceptionally high as compared with scores of other experiments for the same length of time. Usually such an exposure is followed by not more than 3 per cent. of swimming larvæ but many times swimming forms do not appear at all.

20, 30 or 40 minutes, quite a goodly percentage of larval forms are to be seen, but compared with the curve of larval production of the hypertonic solution alone it has been increased very little or not at all. We cannot, therefore, refer the swimming larvæ appearing in these inseminated cultures to fertilized eggs but they can be regarded only as larvæ that have developed from the action of the hypertonic solution upon the eggs. Insemination did not increase the number of cleavages or swimming larvæ over that obtained after hypertonic activation alone.

We can now consider more in detail the results of the experiment under consideration and let it serve to illustrate the results of most of the experiments.

In an exposure to the weaker concentration of hypertonic sea-water very few eggs give evidence of a cortical change that in any way resembles the fertilization membrane produced around the egg when normally fertilized, and in keeping with this, very few of the eggs segment. It is difficult to say whether or not every egg that cleaved had previously undergone a cortical change. It is difficult even to say if an egg has cleaved or not, for the cleavage patterns have been so variously modified, are so irregular and these are scattered promiscuously among so many eggs that are undergoing degeneration that it is very difficult to determine whether an egg has segmented or is slowly falling to pieces. Many times the cytoplasm of the egg pulls apart, leaving a wedge-shaped opening that finally separates the egg into two approximately equal halves with the nucleus contained entirely within one half; and yet at other times nuclear division may occur without cytoplasmic division. But that there is not a membrane produced that will hold the blastomeres together as in normal fertilization is very evident from the fact that even the first two cells may fall apart and become entirely separate from each other or be held together only within the clear transparent jelly layer of the egg—the chorion layer. But a small percentage of the eggs do produce slight membrane changes: the membrane appears around the egg and can be easily seen with the higher powers of the microscope. They are slightly thickened, clear jelly-like membranes adhering very close to the egg and are not at all to be confused with a normal

membrane. It is therefore possible to assume that all eggs that have segmented have been preceded by cortical changes of some intensity at least.

As one may see from Table I., the largest percentage of cleavages obtained from an exposure to the weaker hypertonic solution (Lot A) was 8 per cent. and that the greatest number of swimming larvæ was 2 per cent., therefore many of the eggs had segmented that did not continue their development to the swimming stage. The optimum effect of the solution for initiation of development was not imparted to the eggs and development ceased after a few cleavages. In the great majority of eggs, however, no evidence of initiatory changes are discernible and one would naturally suspect that they were yet capable of fertilization, and this, as the experiment shows, is partially true. The shorter exposure eggs when inseminated immediately after return to sea-water, react quite normally with sperm, producing a fertilization membrane, normal cleavage and a high percentage of swimming larvæ. But as the exposures are lengthened there is a gradual decline of the normal conditions until an exposure to hypertonic solution, sufficiently prolonged, will entirely prohibit fertilization of the eggs.[1] After an exposure, lasting 25 to 30 minutes, to this concentration, the eggs begin to show a tendency for loss of pigment: they are lighter in color and a few are so affected as to be reduced to mere shadows of their former condition, but this is much more true of the longer exposures.

The character of cleavage and of larval development also declines from the normal as the curve of fertilization approaches zero. In the experiment cited, dishes A 1 and A 2 gave practically normal cleavage and 85 per cent. to 95 per cent. of swimming forms that appeared practically normal. Beginning with dish A 3 however and extending throughout the series of exposures, cleavage became less and less normal; the cleavage planes had grown more indistinct and in many eggs division of the nucleus had gone on while the cytoplasm remained unsegmented. Some of the dishes presented such an indistinct lot of cleavages that it was almost impossible to judge whether or not an egg

[1] Lack of fertilization after exposure to solutions of various salts, has been pointed out by R. S. Lillie, '11.

had cleaved but the cleavage count is as nearly correct as very careful examination could make it. The larvæ appearing in dish A 1, after insemination, were a good lot: approximately 70 per cent. swam at the top of the water and more than 50 per cent. are entirely normal. Compared with the swimming forms of A 5, obtained after fertilization, there is a great difference: only a small percentage of the eggs subjected to the hypertonic solution for one hour (A 5) and inseminated immediately, gave rise to swimming larvæ; and of these only a few could be seen swimming at the top of the water. Almost any form of moving masses of protoplasm may be found among the forms of this dish from a very few apparently normal forms to masses that gave only the least bit of quivering movements; blunt masses, angular forms possessing no semblance of morphological similarity to a normal form, dumb-bell shapes one half of which swims and pulls the other lifeless half around with it, bits of spinning protoplasm that appear somewhat as half, quarter or eighth blastulæ are to be found abundantly. As the length of exposure to hypertonic solution is increased before insemination one encounters a more and more abnormal lot of larvæ; there is not only an increase in the degree of abnormality but the percentage of swimmers is gradually reduced until dish A 11 (inseminated) contained only 10 per cent. of eggs that gave the least signs of life, and of these not any swam at the surface of the water; A 12 (inseminated) revealed not a single form that approached the normal.

It is well to point out again the inability of one to obtain an absolutely correct larval count, for among the eggs that have not been fertilized are many that have undergone cytolysis; the count is only relatively correct but it will be sufficient to illustrate the decrease of the capacity for fertilization of eggs that have been exposed to the activating influence of the weaker solution of hypertonic sea-water.

When the more concentrated solution is used as an activator and is followed by insemination, a different condition is encountered. After the hypertonic treatment alone, of say thirty minutes, one obtains a much larger percentage of cleavage and a decidedly higher percentage of swimming larvæ than is

obtained after the same exposure to the weaker solution. But a comparatively short exposure (5 to 10 min.) to the stronger concentration results in the production of quite a considerable number of membranes. The membranes too are decidedly more pronounced than those produced after exposure to the weaker solution but are not yet like the fertilization membrane produced in normal fertilization, even though the physiological processes responsible for their production may be analogous except perhaps in intensity. Cleavage, after this exposure, although departing very greatly from the normal, is not characterized, as are many of them after the weaker solution, by the separation of the blastomeres; the cells remain in close proximity to each other—a fact that may possibly be associated with the more rigid membrane that is produced around the egg—and a larger percentage of them reach the swimming stage than of those from the other solution. But here also as the length of exposure is increased and the curve of cleavage falls, the same decline in degree of normality and of percentage of swimming larvæ is apparent.

In keeping with the more pronounced effect of the stronger solution in the production of membranes is the fact that the eggs suffer a greater increase in permeability. Loss of pigment is more pronounced than in the case of the weaker solution as one would expect. A small percentage of swimming larvæ obtained after a relatively short exposure to the stronger solution are somewhat normal, but the lots grade so gradually into abnormality and immobility that many can be seen to possess but very little power of movement. Many half, quarter or eighth sized larvæ are found here, as well as following an exposure to the weaker concentration.

There is revealed here a general shake-up of development that is quite characteristic, not merely for the hypertonic treatment, but for development after treatment with any agent that tends to destroy the delicate physico-chemical balance of an egg.

This experiment which may be considered a typical one, offers no indication that the addition of a sperm suspension to eggs that have been activated by hypertonic sea-water are yet

capable of being fertilized, but on the contrary many eggs that have failed to respond outwardly to the activating influences, by segmenting, are found to be incapable of fertilization. The reaction differences of the eggs or their different physiological states assert themselves by the variety of reactions given by the egg after insemination; one egg will segment and produce a swimming larva; another will divide a few times but finally disintegrate; yet still another will remain to all appearances in a normal condition, being normal in size and color, will be surrounded with spermatozoön and yet does not divide; while finally one will retain its full rounded appearance but give up practically all its pigment content to the surrounding water, but does not segment. If the eggs are given the optimum exposure for cleavages,[1] insemination does not increase either the percentage of swimming larvæ or of cleavages.

Professor Loeb, however, makes the claim that sea-urchin eggs may undergo activation, from an exposure to hypertonic sea-water, to such an extent that they segment once or twice, as the case may be, but are nevertheless still capable of fertilization. Quoting from Loeb:[2]

"When we put the unfertilized eggs of *Strongylocentrotus purpuratus* directly into hypertonic sea-water . . . and if we put them back at different intervals into normal sea-water, we find that if eggs have been exposed a sufficiently long time (two hours or more) to the hypertonic sea-water a number will begin to segment. These eggs will often go into the two- or four-cell stage, or sometimes to the eight- or sixteen-cell stage, and then stop developing . . . such eggs remain after this perfectly normal and they have the appearance of small unfertilized eggs. If we wait for sometime, say twenty-four hours, to make sure that they neither develop nor disintegrate, and add sperm, each one of these blastomeres forms a tightly fitting membrane. They begin to develop in a perfectly normal way and into normal larvæ."

Whether or not the fundamental principles of development in the egg of *Arbacia* differ from those of *S. purpuratus*, the

[1] In this particular experiment an exposure to the stronger solution for 30 minutes.

[2] " Art. Par. and Fert., p. 237.

results obtained by the writer with the eggs of *Arbacia* do not agree with the observations of Professor Loeb on the eggs of the California sea-urchin.

The eggs of *Arbacia* will, as does the above cited instance of behavior of the *Stronglyocentrotus* egg, segment into two or four cells and go no farther in development, but remain for several hours apparently normal half or quarter eggs. This condition is perhaps best obtained in *Arbacia* following a hypertonic treatment of thirty to forty minutes, with the weaker solution. If such a lot of eggs are returned to normal sea-water and allowed to remain at room temperature (21°–23° C.) for twenty-four hours, many of them will appear quite normal, while all gradations of loss of pigment can be found. There are present also many small eggs of half or quarter normal size but the majority of these are not normal; most of them are entirely or partially devoid of pigment; some few of them however do appear normal in color. If sperm are added to such dishes and the effects of the insemination carefully noted, a few very striking facts are to be observed:

(1) The smaller eggs as well as the eggs that have not segmented are surrounded by actively swimming spermatozoa but they do not produce a fertilization membrane;[1] (2) many of the unsegmented eggs will segment, but the blastomeres are not held together. Since a good membrane is not produced, the blastomeres may become entirely separated from each other or be retained within the very thin jelly layer of the egg and not be in contact with each other as they are normally. These blastomeres may again divide giving rise, each, to two daughter blastomeres that may again become separate, making four individual blastomeres from the same egg or four quarter-eggs; and these may go on dividing until a great mass of cells are present but not held together. Some few of them (for instance all those derived from one of the first two daughter cells) may remain in loose approximation and perhaps give rise to a very

[1] In many cases eggs that have been exposed to the weaker concentration for a short time only, will produce a quite typical membrane after insemination, but those exposed for one hour to this solution, and allowed to remain standing for twenty-four hours, are almost entirely devoid of any semblance of membrane production after insemination.

abnormal and much smaller swimming larva than the normal. On the other hand, the writer has watched for long periods of time the half- or quarter-eggs found in the dish at the time of insemination but has not found for certain a single one of these that produced a membrane or has segmented as a result of fertilization. But if one should only casually observe the eggs following such a treatment there is no way to distinguish between the separated blastomeres of an egg that has been fertilized and has segmented into two blastomeres that have become separate, and one of the half- or quarter-eggs that was present at the time of insemination. Of importance also is the fact that the *character of a swimming larva of reduced size found in these inseminated cultures is no criterion that would indicate from what kind of an egg it was derived.* As before stated, swimming larvæ of all sizes and of most any external shape assumed by protoplasm can be found in dishes, the eggs of which have been exposed to the hypertonic treatment alone. How then may we conclude that a half- or quarter-sized larva obtained in such a mixed culture after insemination, was derived from a half or quarter-sized egg by fertilization? The only possible way in which the writer could know the exact conditions of the egg from which the swimming larvæ came was by the very laborious method of isolation of individual eggs, and this method was adopted as a last resort.

Four or five hours after an exposure to the weaker concentration of hypertonic sea-water one often finds that 2 per cent. or 3 per cent. of the eggs have segmented once, producing two equal blastomeres that are separated from each other but remain enclosed by the thin transparent jelly layer of the egg. By means of a very fine capillary pipette connected with a flexible rubber tube held in the mouth, these two separate cells, or half-eggs, were isolated from the cultures while in focus under a binocular microscope and collected into a small dish. Thus it was definitely known that only blastomeres of one kind were present. These isolated half-eggs could then be observed and be inseminated in a pure culture, and hundreds of them were isolated and studied in this manner, enabling the writer to determine the following points:

(*a*) Not a single blastomere of the first cleavage of an egg (a half-egg) isolated and left standing in normal sea-water at room temperature, appeared entirely normal at the end of twenty-four hours.

(*b*) A few of these blastomeres, without further treatment, segmented again and some many times. This cleavage was often delayed some hours after treatment, indicating that developmental processes were going on very slowly.

(*c*) Not a single normal larva was obtained out of hundreds of these isolated blastomeres inseminated seven hours or longer after the treatment.

(*d*) These inseminated, apparently normal blastomeres did not form a fertilization membrane.

(*e*) Out of the hundreds of half-eggs that have been inseminated less than 10 per cent. have ever segmented. It is just possible that some of these cleavages have been due to activation by spermatozoa, but the eggs had been so weakened that cleavage was abnormal and never reached the swimming stage. *But beyond doubt most of these cleavages were due to the slow development going on within the egg similar to* (*b*) *above.*

With these facts in mind is it not possible to account for the results of Loeb without considering that his half- or quarter-larvæ came from the fertilization of the separated blastomeres of the 2- and 4-celled stage? As pointed out above, eggs that have remained standing for 24 hours after hypertonic exposure do not produce membranes after insemination, that are rigid enough to hold the blastomeres together; and in these experiments, as well as in Loeb's, many dwarf and badly deformed larvæ can be seen in the culture but they do not come from the fertilization of a half- or quarter-egg, but rather from an egg that was fertilized and later lost cells. Numbers of swimming forms can be seen trailing a comparatively huge mass of cells after it, that have been lost from the egg itself and consequently has resulted in a reduction in size. Even in the cultures producing swimming larvæ from the effects of the hypertonic solution alone, one encounters these half-, quarter-sized, and even smaller swimming masses representing parts of eggs, but these too are reduced in size from loss of cells during the division of a

whole egg and possibly also from an egg, one half of which may have become separated after the first cleavage.

The fact that among these 24-hour cultures of eggs, insemination is not usually followed by a perceptible membrane, may lead one to consider the second division of an egg as a division of two half-eggs that have segmented since insemination but not as a result of it. If these first two blastomeres from the cleavage of an egg are separated and each divides again, how is one to judge whether this is the real condition or that perhaps they may represent the fertilization of a half-egg? Decidedly not by periodical examination, but only by continual observation, if in a mixed culture, or by isolation; and as has just been pointed out, hundreds of isolated blastomeres have been inseminated in pure culture but not a single swimming form has been obtained from these, that have remained at room temperature seven hours between the time of hypertonic treatment and insemination.[1] Whether this explanation will serve to harmonize the differences of opinion or whether there is an individual variation between the fundamental processes of development in the two different species of sea-urchin eggs, the writer is unable to say.

But to obtain a further insight to the observable effects of the addition of sperm to eggs that have been caused to start their developmental processes, eggs from a large number of experiments have been preserved and sectioned and the cytological results are presented briefly in a following section.

2. *Fertilizin and Fertilization.*

In a former paper the writer considered in some detail the relation of the presence of the sperm agglutinating substance, fertilizin, liberated from the normal eggs of arbacia into seawater in which they have been standing, and the capacity of eggs for fertilization, both normal eggs and eggs that had been treated with various reagents. It is not the purpose of the

[1] Some time must be allowed before insemination because of the fact that some of these blastomeres continue to divide before insemination; even seven hours does not entirely eliminate this source of confusion, for a blastomere may divide after that length of time. Developmental changes are still going on, but at a very slow rate.

present paper to take up for discussion the disputed points of the fertilizin theory[1] nor to discuss the way in which fertilizin acts or its rôle in the process of fertilization, but it is perhaps desirable to add another instance of the existing parallelism between the presence of a detectable amount of this agglutinating substance and the capacity for fertilization. Professor Lillie and the writer have previously shown this parallelism to exist in all cases thus far tested: (1) Normal, ripe *Arbacia* eggs liberate this substance into the sea-water as long as they are capable of fertilization; eggs whose germinal vesicles have not broken down do not liberate fertilizin in detectable quantities and they cannot be fertilized. (2) If *Arbacia* eggs have been exposed to the optimum concentration of butyric acid and full membranes were produced, no fertilizin was detectable and the eggs could not be fertilized. (3) If eggs are over-exposed to the same concentration of butyric acid (1 to 3 mins.) the eggs can be, at least, partially fertilized and fertilizin is readily detected. (4) If they are exposed for two hours to this same concentration of butyric acid (50 c.c. sea-water + 2.8 c.c. N/10 butyric acid) they cannot be fertilized and fertilizin is not liberated in detectable quantities. (5) If eggs are exposed to heated sea-water of 35° C. for 10 minutes they could not be fertilized nor could fertilizin be detected; and finally to be added from these experiments. (6) If eggs are exposed to a hypertonic sea-water solution for one to one and one-half hours, sometimes some of the eggs are still fertilizable to a limited extent but always such conditions have been accompanied by a detectable amount of fertilizin; but in conditions where the eggs are not capable of fertilization no fertilizin has ever been detected. Only one experiment will be given in detail to show this parallelism (see Table II.).

From microscopical examination at 5:00 P.M., August 15, one could see that many eggs of the X lot were apparently normal in appearance; they were well rounded, and seemed to possess practically the normal amount of pigment, but upon insemination they did not produce fertilization membranes and the blastomeres after segmentation were not held together, and not one of them developed to the swimming blastula stage.

[1] See Lillie, '14, Moore, '16.

Experiment: Aug. 14, 1916.

2:30 P.M. Eggs collected and washed—divided into lot X and lot Y.

3:05 P.M. X put into (50 c.c. sea-water + 8 c.c., 2½ m. NaCl). Y put into (50 c.c. sea-water + 16 c.c., 2½ m. NaCl).

4:25 P.M. Both lots returned to sea-water and lightly shaken to free from jelly, and series of washings begun.

TABLE II.

Time.	Designation.	Lot X.	Lot Y.
4:50 P.M.	Washing 1	$\frac{10}{1.0}$[1]	$\frac{10}{1.0}$
5:10 "	" 2	"	"
5:50 "	" 3	"	"
6:00 "	Inseminated	Cleavage 10 per cent.	0
Aug. 15			
8:30 A.M.	Washing 4	$\frac{10}{1.0}$	$\frac{10}{1.0}$
9:10 "	" 5	"	"
10:45 "	" 6	"	"
11:10 "	" 7	"	"
12:30 P.M.	" 8	"	"
2:10 "	Tested for fertilizin	8 second reaction	negative
	Inseminated	Cleavage 13 per cent.	0
2:15 "	Washing 9	$\frac{10}{1.0}$	$\frac{10}{1.0}$
3:00 "	" 10	"	"
3:20 "	" 11	"	"
4:00 "	Test for fertilizin	5 second reaction	negative
4:20 "	Washing 12	$\frac{5.0}{1.0}$	$\frac{5.0}{1.0}$
5:00 "	Tested	4 second reaction	negative
	Heavy insemination	Cleavage 20 per cent. (poor)[2]	0
5:20 "	Eggs killed in Boveri's picro-acetic acid		

The results of this experiment have been confirmed by a number of others and the results have always been consistent—if the supernatant fluid possessed enough fertilizin to cause the agglutination of a fresh sperm suspension, always there had been eggs present in the culture that possessed enough latent developmental capacity to react with a spermatozoön and to segment as a result of this union, but if no fertilizin could be detected never have the eggs been found to possess a capacity for fertilization. Some change in the physiological or physico-

[1] In every case the numerator of the fraction represents the volume of eggs and sea-water while the denominator represents the volume of eggs and sea-water (in c.c.) remaining in the tube after most of the supernatant sea-water had been drawn off—for further details see footnote 1 on page 276.

[2] No larvæ produced from 5 P.M. insemination, in either lot X or lot Y.

chemical state of the egg has been brought about as a result of the hypertonic treatment and fertilization is impossible. Of what this change consists we do not know, but it is at least accompanied by an increased permeability of the egg and loss of substances from it. Whether a specific substance necessary for fertilization has diffused from the egg or whether it has undergone new chemical combinations that have rendered it unavailable and whether it may be the substance called fertilizin are questions that cannot now be answered. But it seems significant that here as in all previously modified conditions there has always been a substance escaping from the egg into the surrounding sea-water that could be detected by its sperm agglutinating properties, if the eggs were still capable of fertilization. That this unfertilizable condition is due to a new physiological state of the egg itself and not to a physical condition of the surface of the egg that might prevent sperm entrance, is made certain by observation on preserved material. Sperm do penetrate these eggs and sometimes produce weak changes similar to fertilization or they remain entirely ineffective, depending upon the condition of the egg itself.

3. *Conclusions of Section III.*

The results of this series of experiments seem to indicate that a certain optimum exposure of the eggs of *Arbacia* to hypertonic sea-water initiates changes within the egg that result in development. That all do not segment is because the optimum exposure for one egg is not the optimum for all.[1] Some have been over-exposed, some under-exposed, but if the exposure was the optimum one, swimming larvæ were produced; if less or greater than the optimum perhaps only one or two cleavages resulted and development ceased; if decidedly over-exposed or under-exposed, cleavage does not occur. This quantitative relation is then interesting from the standpoint of fertilization. If the eggs were not perceptibly affected by the exposure they could be fertilized; if the reactions initiated by the exposure were of sufficient intensity to disturb the physico-chemical condition of

[1] The difference may be due to the factor of aging as Goldfarb ('16) has applied the term.

the egg and cause the loss of substances necessary for fertilization or has caused new chemical combinations to be formed that renders certain substances unavailable, then fertilization is likewise impossible.

In order to determine definitely whether or not this unfertilizable character of the egg is due to a real physiological or physico-chemical condition of the egg substances or whether it is due to physical conditions that prevent the extrance of a spermatozoön into an egg, a large number of these from different kinds of experiments were preserved and sectioned. Usually Boveri's picro-acetic acid was used as a killer and sections (4 μ thick) were stained in iron hæmatoxylin.

IV. Observations from Preserved Material.

The cytological data to be presented is not meant to be a critical analysis of chromosome behavior but is given only to acquaint us with a few of the more general facts of the behavior of sperm in relation to these unfertilizable conditions, and it will be confined to observations upon three different classes of experiments: (1) To mass cultures of eggs that have been exposed to the weaker concentration of hypertonic sea-water for different lengths of time, returned to normal sea-water and immediately inseminated; (2) to mass cultures which have been exposed to the stronger concentration of sea-water for different lengths of time, returned to sea-water and inseminated; (3) to pure cultures of eggs that have segmented but once after an exposure to the weaker concentration, and inseminated.

1. *Insemination Following an Exposure to the Weaker Hypertonic Solution.*

Experiment:

2 P.M. Eggs were collected, washed three times.

2:50 P.M. Transferred to hypertonic sea-water—(50 c.c. sea-water + 8 c.c., 2½ m. NaCl).

Eggs returned at intervals to sea-water, divided into lots A and Ax. Lot A no further treatment; control.

Lot Ax, immediately inseminated after return to sea-water. Eggs from both lots killed as given in Table III.

TABLE III.

No.	Lot A.			Lot Ax.			
	Returned to Sea-water.	Per Cent. Cleavages.	Per Cent. Swimming Larvæ.	Per Cent. Cleavages.	Per Cent. Larvæ.	Insem-inated.	Killed.
1	3:10 P.M.	2	1	85	75	3:15 P.M.	3:45 P.M.
2	3:30 "	2	0	75	75	3:35 "	4:05 "
3	3:50 "	15	1	45	15	3:55 "	4:25 "
4	4:10 "	25	3	30	8	4:15 "	4:45 "
5	4:30 "	12	0	12	1	4:35 "	5:05 "
6	4:55 "	20	1	12	2	4:55 "	5:25 "

Microscopical Observations on Lot A.

3:30 P.M., A^1 eggs appear normal.

3:55 P.M., A^2 eggs appear normal.

4:15 P.M., A^3 eggs appear normal.

4:40 P.M., A^4 10 per cent. eggs show loss of pigment.

5:10 P.M., A^5 few eggs almost colorless; 30 per cent. appear normal.

5:20 P.M., A^6 some eggs reduced to shadows, many appear quite normal.

Sections of eggs of the AX^1 lot appear not greatly different from lots that have been normally fertilized; very few of them

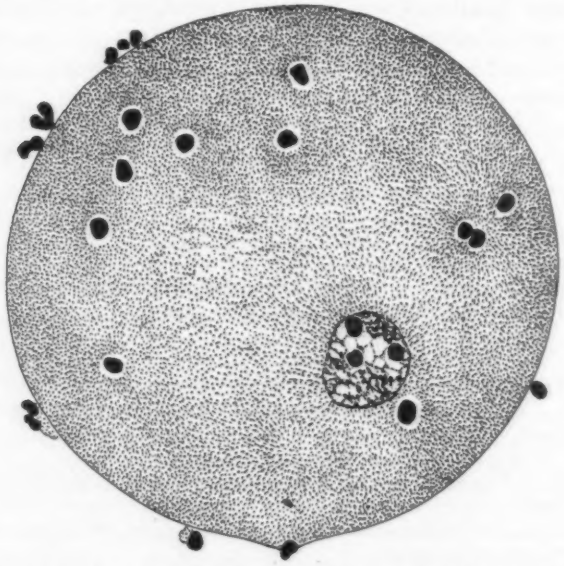

FIG. 2. × 3,000. Section of an egg exposed to the stronger hypertonic solution for 15 minutes and inseminated immediately on return to sea-water. Killed 15 minutes after insemination. The sperm heads are entirely unchanged and have not reacted with the egg.

show more than one sperm nucleus and this has usually undergone the characteristic swelling and vacuolization changes of a male pronucleus and is accompanied by an aster. After a longer exposure to this concentration of hypertonic sea-water the resemblance to sections of normal fertilization gradually disappears and is superseded by a general condition of polyspermy and later by a non-reactive state of the egg substances,

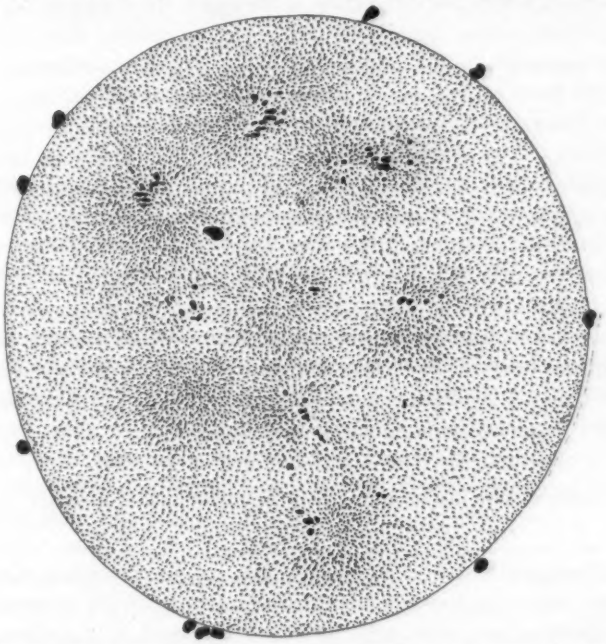

FIG. 3. × 3,000. Section of an egg exposed to the weaker hypertonic solution for two hours and inseminated immediately on return to sea-water. Killed 20 minutes after insemination. All but one of the sperm heads are undergoing fragmentation. The egg nucleus does not appear in this section.

with an abundance of spermatozoa, lying within the cytoplasm, entirely unchanged. Of the eggs of AX^3, probably the larger percentage are polyspermic and show a reactive condition of the sperm heads; they appear swollen and slightly vacuolated and are usually accompanied by an aster more or less well formed. Some sections of this lot still appear almost normal in their

general characteristics. In lot AX[5] however some of the sperm heads are slightly swollen but are not associated with astral radiations and still others are surrounded with weak radiations. Many cases are encountered in which the egg nucleus has broken down into chromosomes, dispersed along the rays of a unipolar spindle, formed by the egg, as a result of the hypertonic treatment; and at the same time several sperm nuclei may lie unchanged within the cytoplasm of the egg.[1] In AX[6] most of the eggs have almost completely lost their capacity to react with a spermatozoön. The majority of the sections show the sperm heads entirely lacking an aster, but many asters produced by the effects of the hypertonic solution are present and are very definite. The reaction between the egg and sperm is largely negative. In numerous sections the physiological changes within the egg cytoplasm have not only prevented changes characteristic of normal fertilization, but they are even of such a character as to cause the disintegration of sperm heads. Fig. 3 is a section of such an egg and shows one sperm head yet unchanged but several others are undergoing dissolution. Other sections show another type of disintegration which is characterized by a swollen vesicle about the sperm head which is breaking apart and stains very faintly with iron-hæmatoxylin.

2. *Insemination Subsequent to an Exposure to the Stronger Solutions.*

The examination of sections of eggs of the following experiment serves to give a general understanding of the incapacity for fertilization, after eggs have been exposed to the stronger solution of hypertonic sea-water.

Experiment:

4:30 P.M. Eggs collected, washed three times.

5:10 P.M. Transferred to hypertonic sea-water—(50 c.c. sea-water + 16 c.c., 2½ m. NaCl).

Eggs transferred at intervals to sea-water and divided into two lots X and Y. Lot X remains standing without further treatment. Lot Y inseminated immediately after return to sea-water. Eggs were removed from each lot at definite times and preserved as given in Table IV.

[1] See Wilson, '01, and Hindle, '10.

TABLE IV.

No.	Returned to Sea-water	Lot X.		Lot Y.	
		Percentage of		Percentage of	
		Cleavages	Swimming Larvæ.	Cleavages.	Swimming Larvæ.
I	5:15 P.M.	10	0	25	3
II	5:20 "	6	0	12	7
III	5:25 "	5	2	12	3
IV	6:00 "	20	2	22	2

Series Preserved in Picro-acetic Acid.

I x killed 5:30 P.M.	I y^1 killed 5:30 P.M.	I y^2 killed 5:45 P.M.
II x " 5:50 "	II y^1 " 5:35 "	II y^2 " 5:50 "
III x " 5:25 "	III y^1 " 5:40 "	III y^2 " 5:55 "
IV x " 6:15 "	IV y^1 " 6:15 "	IV y^2

The general cytological observations on this preserved series are as follows: In all lots of eggs exposed to the stronger concentration, from five minutes to fifty minutes, and inseminated immediately upon their return to sea-water, sperm have penetrated the eggs freely and lie in any plane of the egg cytoplasm from the periphery to the center. No attempt has been made to determine by actual count, from sections, the relative proportion of eggs containing spermatozoa, but this condition is found under every field of the microscope and many sections of eggs show from two to sometimes twenty sperm heads. By far the larger number of eggs show the presence of sperm heads imbedded within the cytoplasm.

The effects of the egg cytoplasm environment upon the individual spermatozoön varies in the different eggs in which they are found. Many sections prove by their presence that three, four or more spermatozoa have entered the egg and react with it, thus giving the picture of a typical case of polyspermy; the sperm heads may be separated from the egg nucleus and accompanied by a sperm aster, or two or three may have copulated with the egg nucleus. In other sections a dozen spermatozoa may be scattered throughout the cytoplasm, but thirty minutes after insemination they exhibit not the slightest perceptible change in size or shape (see Fig. 2). They appear perfectly solid with no indications of vacuolization; there is no indication of a sperm aster, and in short the sperm heads appear only as inert foreign

bodies in the cytoplasm of the egg.[1] Still other sections exhibit a different type of reaction of the sperm to its new environment, that appears significant—the sperm heads may undergo swelling and vacuolization without any indication of the appearance of a sperm aster. For the most part asters arise only in connection with a spermatozoön that shortly appears to be in the swollen, rounded, and slightly vacuolated condition but, as has been mentioned, some possess much more pronounced astral rays than others. This condition of the entire absence of a sperm-aster from the spermatozoön undergoing the more or less characteristic changes of activity, probably to be referred to chemical influences exerted upon it by some substance within the egg, seems to indicate a quantitative reaction of the spermatozoön with the egg, the sperm aster formation being the more sensitive factor. In fact this series of preparations exhibits a gradient of the effects of a spermatozoön, from essentially its normal effect, to a condition in which it provokes not the slightest change; and since a sperm aster is usually associated with a swollen sperm head cannot this be an indication of the suppression of sperm aster formation but a continuation of the more stable changes accompanying sperm activity?

(*a*) *Penetration of the Spermatozoön.*—An interesting condition for the observation of sperm entrance is presented by this series of preparations and since the writer knows of no observations on sperm entrance in *Arbacia* some passing mention of it may be of interest. As may be seen from Fig. 4, two spermatozoa are in the act of entering an egg and many other such sections are to be found among the series. The egg has produced a small protrusion of protoplasm as an entrance cone that projects slightly above the egg surface; and within this cone lies the sperm head pulled out into a narrow chromatin band, one end of which projects down into the cytoplasm. No definitely formed fixation body, to which the inner end of the spermatozoön is attached, has been observed but the very shape and character of the entering spermatozoön would indicate a decided attraction from within the egg that acts upon the sperm head.

[1] Normal sperm asters appear within five to ten minutes. Around most of the sperm heads is a small clear area produced by the retreat of protoplasmic granules of the egg from its periphery.

Fig. 4 was made from lot III Y[1] (page 283) and perhaps cannot be considered as representing the normal mode of entrance of the spermatozoön of *Arbacia* but it at least indicates the possible method of entrance. It may be necessary to attribute the appearance of the phenomenon to the general retarded condition of the egg protoplasm brought on as a result of the hypertonic treatment before insemination, but it would not seem impossible to discover a somewhat similar condition of sperm

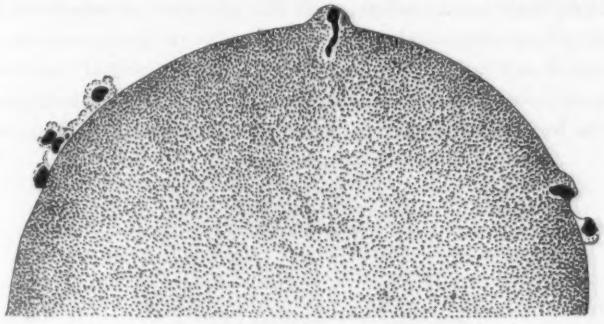

FIG. 4. × 3,000. Part of a section of an egg showing the entrance of the spermatozoön; one has practically completed its entrance and is rounding up, the other in the process of entrance. From same lot as Fig. 2.

activity under normal conditions if the right stages were preserved in a close enough series. Penetration, in *Arbacia*, is effected much more readily than in *Nereis*, in which Professor Lillie[1] has illustrated so clearly the entrance of the spermatozoön. The most essential difference in the morphology of the process in the two forms is the absence of the attraction cone in *Arbacia* which in *Nereis* forms an attachment for the spermatozoön and apparently pulls the spermatozoön through a small aperture in the vitelline membrane as it recedes from the periphery of the egg. Immediately after the spermatozoön is taken inside the egg it assumes its normal appearance, for no elongated heads have been observed that were not in the act of entering the egg.

3. *Insemination of Pure Cultures of Isolated Blastomeres.*

Since the experimental results of the writer did not agree with the views of Loeb—namely, that eggs whose development

[1] See Lillie, F. R., '11 and '12.

had been initiated by an exposure to hypertonic sea-water, could yet be fertilized, produce fertilization membranes and develop into swimming larvæ, he was anxious to know if fertilization was prevented by failure of the sperm to enter or whether the individual blastomeres—the products of the first cleavage of an egg—had developed an immunity to the latent effects of a spermatozoön.

It is obvious that to determine this point from cytological preparations a pure culture of the first two blastomeres was highly desirable; and with considerable difficulty some two hundred and fifty or three hundred eggs that had segmented but once were isolated from lots of eggs that had been subjected to the weaker hypertonic solution. This pure culture of approxi-

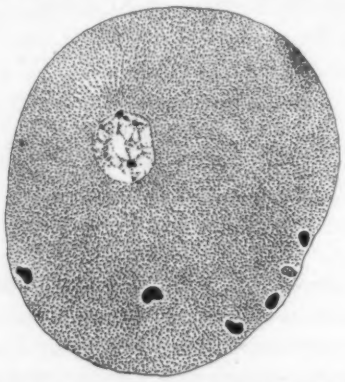

FIG. 5. × 3,000. Section of a quarter egg showing the presence of sperm nuclei. Weaker hypertonic solution, 30 to 40 minutes; egg segmented once, isolated, each blastomere segmented again. Killed 20 minutes after insemination.

mately five hundred half-eggs was heavily inseminated, allowed to stand for twenty minutes and preserved in Boveri's picro-acetic acid. A very few of these blastomeres had divided again into two daughter blastomeres (quarter-eggs) before the lot was inseminated, but every egg was isolated from the mass culture into a pure culture while in the two-celled stage.[1]

Microscopical sections of these half-eggs prove to us beyond

[1] As a definite membrane was not produced by the egg following the hypertonic treatment the blastomeres became entirely separated from each other in the pure culture. All were half-eggs when isolated.

any possible doubt that sperm do enter the blastomeres (Fig. 5) but the normal effect of a spermatozoön entering an egg is not apparent. Many of the sperm heads lie within the cytoplasm entirely unchanged, while others are seen to have increased in size from swelling, but most of these are undergoing evident degeneration, of which various stages can be seen (Fig. 6). The chromatin gradually loses its staining reaction, appearing much clearer than the unchanged sperm heads outside of the eggs, and some have so entirely lost their staining capacity that they appear only as clear vacuoles scattered throughout the cytoplasm of the half-eggs. Out of the vast numbers of sperm heads observed within these half-eggs only one was found that contained an indication of a good sperm aster. A few of the

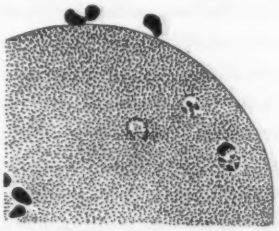

FIG. 6. × 3,000. Part of section of a quarter egg showing presence of both unchanged and degenerating sperm nuclei. No indication of fertilization changes. Same exposure as Fig. 5.

blastomeres are undergoing segmentation as a result of the hypertonic treatment and the spindles appear quite normal.

Here then the spermatozoön not only does not find a congenial environment in which normal reactions can go on, but the surroundings are even hostile and result in the disintegration of the spermatozoön without its having undergone any changes that indicate the least possibility of a fertilization reaction.

4. *Conclusions of Section IV.*

This cytological examination very clearly reveals the fact that the unfertilizable condition of eggs subsequent to an exposure to hypertonic sea-water is not due to the failure of a spermatozoön to enter the egg but that it is due to a change within the egg itself. Certain changes that are to be associated

with initiation of development have produced an environment in which the reaction between a spermatozoön and an egg has been rendered impossible. *Spermatozoa enter the eggs in comparatively large numbers but the eggs are not fertilized.*

In the shorter exposure to hypertonic sea-water many eggs do react with a spermatozoön but since these are conditions in which a very small percentage of eggs segment and a still smaller percentage reach the swimming stage after the hypertonic treatment, the observations are interpreted as indicating an effect of the hypertonic solution below the optimum exposure for the initiation of development and as a consequence the eggs have experienced but little change from the normal condition; the exposure did not lead to the initiation of development, therefore the eggs still possess their capacity for fertilization. That this lack of a capacity for fertilization is one that is gradually developed as the exposure to the hypertonic solution is increased, is one of the factors that indicates the quantitative side of fertilization. After a short exposure, polyspermy, in which all sperm seem to have a part, is the general rule; a more pronounced effect of the hypertonic sea-water results in a greater number of sperm entering the egg, some showing very weak asters, others being only slightly swollen, while yet a more decided influence may create an environment within which the spermatozoön is not only inactive but also it may even undergo disintegration. Whatever changes have resulted from treatment with hypertonic sea-water, spermatozoa that gain entrance to eggs that have been exposed to optimum conditions for segmentation, or that have been decidedly over-exposed, do not meet with an environment that will permit of a reactivation of the egg.

This degeneration of spermatozoa in a non-reactive egg environment calls to mind essentially similar conditions, encountered normally, in the fertilization of meroblastic eggs such as the pigeon egg described by Blount ('07). Polyspermy in meroblastic eggs appears to be normal but the accessory spermatozoa, after the environment has been changed by reactions within the egg set up by a reaction of the egg with the successful spermatozoön, are repelled peripherally and degenerate and disappear.

V. Discussion.

For an egg to develop, some stimulus must initiate certain changes within it which, having been once set in motion, appear to be autonomous. If an egg is not fertilized, or its development initiated by some artificial agent, it normally does not develop. But studies in the field of the artificial initiation of development have brought to light many methods that may be employed successfully to set in motion the developmental mechanics latent within the egg. As yet we are unable to determine just the mechanism by which these agents are effective, but practically all methods agree in one essential at least—the permeability of the egg membrane is increased.

Practically every one is agreed that the egg must be considered, a very delicately adjusted physico-chemical system, within which, if a spermatozoön gains entrance, a characteristic series of reactions are set in motion that lead to development and a consequent change of the system. If artificial agents are employed as a substitute for the spermatozoön not only do we have conditions changed but also if the proper procedure is known and applied the end results are essentially the same as when initiation was effected by a spermatozoön, *e. g.*, cleavage, gastrulation and swimming larvæ. After the initiatory changes have been induced a spermatozoön in either case is entirely unable to produce any of its characteristic effects.

The delicately adjusted condition of the egg is only a temporary thing for if sea-urchin eggs are allowed to stand in sea-water for a day or more they gradually lose their power of fertilization. Not only can this unfertilizable condition, accompanied by increased permeability, be produced by standing but also by hypertonic sea-water, butyric acid, by a slight rise in temperature of the sea-water and several other agents including, of course, fertilization.

Many writers have considered an unfertilizable condition as an indication of the death of the egg but to cite only one instance to disprove this, the writer has called attention to the fact that if initiation of development is partially accomplished by a treatment with butyric acid and the membranes so formed are destroyed by shaking, sperm will enter the eggs of *Arbacia* but do

not assert any capacity for the initiation of development: however if the eggs were subsequently exposed to the proper treatment with hypertonic sea-water a goodly per cent. will develop to swimming plutei. The non-fertilizable condition is not due to the death of the egg but to a changed physiological condition induced by the initiatory action of the butyric acid treatment. Whatever the changes brought about, all of the conditions mentioned above are accompanied by the loss of substances from the egg, if indeed the condition is not a result of the loss of some substance or substances that are necessary for fertilization. This loss may be due either to the escape from the egg of the necessary substance or to new chemical combinations within the egg made possible by the disturbance of its physiological equilibrium.

It is highly interesting to note that a qualitative and possibly a quantitative test for one substance at least has been discovered by F. R. Lillie. This substance fertilizin is continually liberated from the egg into the surrounding sea-water as long as it remains in a fertilizable condition and as pointed out on page 276 it has been found to afford an absolute test (using a fresh sperm suspension as an indicator) of the capacity of sea-urchin eggs for fertilization in seven analyzed cases and will probably be found to hold for all cases in *Arbacia*, as it is evidently an indicator of a generalized condition.

An analogous condition to that in *Arbacia*, of the loss of substances from the egg and the consequent lack of capacity for fertilization, has been found in other forms than the sea-urchin, though in no other forms than *Arbacia* and *Nereis* has a qualitative test for a substance been found. E. E. Just ('15) has discovered that if eggs from *Platynereis* are collected in sea-water they cannot be fertilized. This is due to some effect of the sea-water in causing the loss of necessary substances for fertilization either through diffusion from the egg or through new chemical combinations within the egg. If only a few drops of sea-water come in contact with the egg they will not fertilize despite the fact that sperm penetrate the eggs and produce weak changes characteristic of fertilization: the proper egg substances for the intiatory reactions are not available.

Miss Allyn ('12) found that any substance that had a tendency to initiate development in *Chætopterus* was detrimental to fertilization. She noted that the tendency was not for the exclusion of spermatozoa but rather for the production of polyspermy. The aberrant type of cleavage became more accentuated as the exposure to the initiatory agent was prolonged and would probably have entirely prevented fertilization with a slightly longer exposure. In this case the initiatory agents induced only a partial completeness of the reactions and as time went on fertilization became less and less possible, indicating the gradual loss of some substance whose presence is indispensable for fertilization. *Arbacia* eggs behave in essentially the same manner if over-exposed to butyric acid; polyspermy appears if insemination is carried out shortly after the exposure, but if the eggs remain standing in sea-water for a few hours insemination has no effect.

Just how hypertonic sea-water is effective we are not able to say, but the writer is inclined to the views of R. S. Lillie that a properly timed exposure to any substance that lowers permeability (unless it be decidedly toxic) allows of the combination of the substances within the egg that starts off the developmental reactions. If these changes are nearly enough the normal, development is quite complete up to certain stages in the life of the organism. The question, however, to which these experiments relate is the capacity for fertilization after activation by hypertonic sea-water.

The quantitative reaction idea has been pointed out quite freely in this and a preceding paper in considering the initiation of development, and certain instances of all phases of this process are to be had not only from the experiments themselves but also from the preserved material. From the purely parthenogenetic standpoint the ideas of the qualitative specificity of hypertonic sea-water as a factor in the initiation of development have been dealt a death blow by the experiments of R. S. Lillie ('15) on starfish eggs, and the purely quantitative idea substituted for the explanation. Lillie finds that not only can starfish development be initiated by a preliminary treatment with butyric acid followed by hypertonic sea-water, but also that a secondary

treatment with butyric acid or warm sea-water or cyanide sea-water may be substituted for the hypertonic sea-water, or that both the preliminary and secondary treatments with warm sea-water may accomplish the same results. From inference any agent or combination of agents that cause a quantitative fulfillment of the reactions lead to the same end.

When a spermatozoön fertilizes an egg the optimum stimulation has been given to the egg, and we assume that the initiatory reactions have been quantitatively complete. But certainly in the initiation by artificial agents some of the eggs will not have been given the optimum conditions and will not have completed their reactions. If we can think in terms of chemical substances we will suppose that part of these have been left uncombined that are yet capable of some reaction with a spermatozoön if it but gain entrance to the egg. Consequently just so far as these fundamental reactions have been incomplete the spermatozoön is capable of asserting its latent stimulus for the activation processes. That fertilization is not complete in such cases is shown by the very irregular type of cleavage and the small per cent. of swimming larvæ and the poor quality of these, just as in a parthenogenetic treatment the same results are encountered if an exposure above or below the optimum one is given. If however the initiatory agent employed, has been affective enough to cause the production of good membranes (butyric acid), or has resulted in segmentation of the egg, the initiatory, reactive, egg substances appear to have been pretty well used up and so far as these experiments show there seems to be no tendency for a regeneration on the part of an egg of the fertilizable condition. The blastomeres of an egg appear to have no capacity, or at the most very little, to react with a spermatozoön. It has been pointed out above that out of hundreds of these isolated blastomeres never has one been able to produce a swimming larva, as a result of fertilization; and sections have revealed but one absolute case of sperm aster formation after penetration. One activation seems to preclude all others.

Fertilization appears to be due to an agent, spermatozoön, that initiates development by producing a condition that permits of the interaction of certain substances within the egg,

which union curtails any further possibility of a subsequent fertilization. If the same conditions are produced by artificial parthenogenesis the same lack of the capacity for fertilization is evident.

Whether the lack of this capacity is due to the absence of one substance or of several substances, and whether the substance called fertilizin is the essential one or is only an indication of a certain physiological state of the egg is unknown. But in every case, so far examined, fertilizin has been present if the eggs could be fertilized and has been absent if there was no capacity for fertilization.

These results are weighty argument, if not definite proofs, against any idea of a sperm-borne substance that is necessary for the initiation of and the continuation of development. There is no evidence that the spermatozoön imparts a "lysin-like" substance that produces cytolysis within the egg and later carries into the egg a "corrective agent" to check the imposed cytolysis. The egg possesses all the substances it needs for development.

VI. Summary.

1. If *Arbacia* eggs are exposed to a weaker and a stronger concentration of sea-water for varying lengths of time a perceptible gradient of effectiveness from the entire absence of any reaction to an essentially normal parthenogenetic reaction is apparent.

2. The effective exposure is accompanied by a perceptible condition of increased permeability and substances escape from the eggs.

3. The superposition of insemination on the optimum hypertonic treatment does not increase the percentage of development.

4. Eggs when exposed to hypertonic sea-water for two hours usually cannot be fertilized; and in such a condition fertilizin cannot be detected by any known method. If however there yet remains a certain capacity for fertilization, fertilizin can be detected by its sperm agglutinating properties.

5. If *Arbacia* eggs are exposed to activating agents the permeability of the egg is increased, substances are lost from it and fertilization is impossible.

6. If eggs are sectioned, after insemination subsequent to an exposure to the weaker hypertonic solution, a gradient of activity of spermatozoa parallels, to a certain extent, the length of the exposure to hypertonic sea-water.

7. If eggs are sectioned after insemination, subsequent to an exposure to the stronger hypertonic solution, large numbers of eggs are seen to have several spermatozoa within their boundaries; but they usually do not exhibit any signs of activity; many are undergoing degeneration.

8. Sperm, in gaining extrance to an egg, are pulled out into a long thin chromatin band situated in the protoplasmic cone produced by the protoplasm of the egg.

9. If a pure culture of the first two cells, produced by cleavage, are inseminated, sperm enter, remain entirely quiescent, and may even undergo disintegration. They do not fertilize the blastomeres.

10. These experiments furnish no evidence for, but are contrary to, any view that a spermatozoön carries a substance into the egg that is necessary for development. There is no evidence of a sperm-borne "lysin-like" substance nor a secondary "corrective agent." The egg possesses all the essentials for development.

HULL ZOÖLOGICAL LABORATORIES,
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THE EFFECT OF STARVATION ON THE WING DEVELOPMENT OF MICROSIPHUM DESTRUCTOR.

LOUISE H. GREGORY.

As is the case with aphids in general, the life history of *Microsiphum destructor*, the green pea-aphid, shows a definite relation to the environment. Dimorphism is common. Winged and wingless individuals appear in varying numbers at different stages in the life history, the wingless forms predominating in the early part of the reproductive history, the winged forms increasing in number toward the end of the summer. While it is generally accepted that external conditions probably control the appearance of wings, very little data has been offered as proof. For a number of years, at the suggestion of Professor Morgan, I have experimented with the effect of starvation on the production of winged forms in the families of the green pea-aphid. I am glad to express my appreciation of Professor Morgan's helpful criticisms.

In starting all experiments, a single individual, selected to produce the stock family, was isolated on a young pea plant in a small pot and covered with a lamp chimney, the top of which was covered with gauze to prevent the escape of the aphid and the confusion of families. This method of isolation was used throughout the investigation and proved most successful, the plants growing normally, sufficient air entering at the top of the chimney. From the family of the stock aphid, some young were isolated and used as control lines, others were removed daily from the plant and starved for periods of different lengths, twenty-four hours, twelve hours and eight hours. Experience proved that fewer deaths resulted if the aphids were starved eight hours daily. After the period of fasting the starved aphids were isolated and their offspring observed. As will be clear later, the effect of starvation on wing development is not apparent in the individuals starved, but in their offspring.

TABLE I.

Starvation Exp. I. — *Control.*

Parent non-winged.
Both series from one parent.
A2 series younger.

Date 1913–14, winter. — 1913–14.

No. Exp. / Hours Starved.	No. Non-winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.	No. Exp.	No. Non-winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.
A1 series 30					Starved—						*Winter*
No. 2.....	14	49	63		4 days—av. 7½ h.	No. 2...	31	14	45		
No. 3.....	6	34	40		per day—	No. 4...	43	4	47		
No. 6.....	14	73	87			No. 6...	28	23	51		
No. 7.....	25	61	86			No. 8...	32	9	41		
No. 8.....	39	37	76			No. 10..	41	3	44		
	—	—	—		Av. family		—	—	—		Av. family
	98	254	352	71+	70+		175	53	228	23%	45·4
A2 series 32¾					4 days starved						
No. 2.....	30	0	30		av. 8.10 daily						
No. 3.....	27	23	50								
No. 4.....	34	40	74								
No. 5.....	23	54	77								
No. 6.....	24	43	67								
No. 7.....	35	43	78								
No. 8.....	16	20	36								
No. 9.....	25	26	51								
	—	—	—								
	214	249	463	53.8	Av. family 57						
Grand Total....	312	503	815	61.1	Av. family 62						

The first experiments were carried on during the winter of 1913–1914 in the greenhouse at Columbia University (Table I.). Five young of a wingless mother were deprived of food 7½ hours daily for four days. These (A1 series) produced a total of 352 young, 254 or 71 per cent. of which were winged. In the second series (A2) younger members of the same family, 8 aphids were starved 8 hours daily for four days. The total number of offspring in the eight families was 463, of which 249 or 53 per cent. were winged. In the control series of a total of 228 (young of five families) 23 per cent. were winged showing a difference of 48 per cent. if compared with the A1 series and of 20 per cent. with the A2 series. If the entire A series is considered as a whole, among the young of 13 families 61 per cent. were winged. Apparently the lack of food has had its effect on the second generation, none of the starved individuals developing wings.

The experiments were repeated at Woods Hole in the summer of 1915 (Table II.). Again the young of a wingless mother were used for the experiment and control. The families of seven wingless females starved 45 hours, were observed and out of a total of 188 young, one winged form only appeared. The seven families of the control series showed 3 winged individuals. Apparently the effect of fasting was overcome in some way.

Considering that the atmospheric conditions had been unusually favorable for the development of the peas, the weather having been very hot with much humidity, I concluded the food had been sufficiently rich to overcome any adverse effects of starvation, and that in order to induce the effects of starvation, it would be necessary to increase the number of starvation periods. Consequently the experiments were repeated the following summer in Princeton, Mass. (Table III.).

Five young starved 60 hours produced a total of 254 offspring, 30 per cent. of which were winged. Ten individuals forming the control series produced 514 young, of which 56, or 9.7 per cent., were winged. Here again starvation has had its effect.

Summarizing all the experiments on wingless parents, it is seen in Table III., that 25 starved individuals produced 1,257 young, 46 per cent. of which had wings: that 22 normal indi-

TABLE II.

Starvation Exp. II. 1915. Parent non-winged.							*Control.* 1915. Parent non-winged.					
No. Exp.	Hours Starved.	No. Non-winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.	No. Exp.	No. Non-Winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.
A_2	45					Woods Hole	A_1					Woods Hole
(1)		37	0	37		Plants luxuriant	(1)	38	0	38		
(3)		15	0	15			(2)	71	3	74		
(4)		18	0	18			(3)	49	0	49		
(5)		32	0	32			(4)	39	0	39		
(6)		36	0	36			(5)	48	0	48		
(7)		18	0	18			(6)	34	0	34		
(10)		31	1	32			(10)	65	0	65		
		187	1	188		Av. family 26.8		344	3	282	1.	Av. family 39.5

viduals produced 1,149 young, 9.7 per cent. of which were winged. The starved mothers had about three times as many winged young as the control. Since all the families were subjected to the same conditions of temperature and humidity, the variation in the results must be due to the only factor in which they differ, namely the food supply.

Temperature undoubtedly may effect the production of wings as Ewing[1] has shown in his experiments with *Aphis avenæ*. He found that by keeping the temperature constant at 65° F., he could prevent the appearance of any winged forms. At lower and higher temperatures winged forms appeared. These results might be interpreted as due *not* to the direct effect of temperature on the development of wings but on the growth of the plants or the food supply and thus indirectly affecting the metabolism of the individuals.

If the summer experiments are compared with those in the winter, it will be observed that the percentage of winged forms is less in both starved and control lines. However the percentage of increase in number of winged forms in the starved lines remains constant. In the summer as in the winter there are three times as many winged young in the starved lines as in the control. I see no reason why the temperature might not have been effective indirectly in the lessening of the wing production in both starved and control summer lines. The atmosphere of the greenhouse was practically constant, while the summer experiments were carried on out of doors and were subject to all the daily changes in temperature and moisture. The actual difference in the development of wings at any one time must have been due to the difference in food supply, temperature having no part. I believe that if aphids were starved for long periods and kept at a constant temperature of 65° F., Ewing's optimum temperature, winged individuals would appear.

Overcrowding on the plants has been suggested as a possible cause of wing development. In nature this might be true, the results however being due to a lessening of the food supply. In my experiments there was no overcrowding as the young were

[1] Ewing, H. E., "87 Generations in Parthenogenetic Pure Line of *Aphis avenæ*," BIOL. BULL., Vol. XXXI., No. 2, 1916.

TABLE III.

Starvation Exp. III. 1916 July. Parent non-winged.						*Control.* 1916 July. Parent non-winged.					
No. Exp. / Hours Starved.	No. Non-winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.	No. Exp.	No. Non-winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.
60 hrs.					Princeton, Mass.						
1.....	35	51	86		Alt. 1100 ft.	2.....	47	0	47		
3.....	23	11	34		Atmosphere dry	4.....	56	3	59		
5.....	50	1	51		and cool	6.....	33	5	38		
9.....	39	14	53		Experiments in	8.....	63	0	63		
11.....	29	1	30		open	10.....	61	0	61		
						12.....	58	0	58		
	176	78	254	30.7	Av. family 50+	14.....	79	11	90		
						16.....	37	35	72		
						18.....	42	0	42		
						20.....	42	2	44		
							518	56	574	9.7	Av. family 57.
Summary											
1913–14					*Winter*	1913–14					*Winter*
13 fam.....	312	503	815	61.7	Starved 30–32 hours. Av. family 62.	5 fam..	175	53	228	23	Av. family 45.
1915					*Summer*	1915					*Summer*
7 fam.....	187	1	188		Woods Hole Starved 45 hours.	7 fam..	344	3	347		
1916					*Summer*	1916					*Summer*
5 fam.....	176	78	254	30.7	Princeton Starved 60 hours.	10 fam..	518	56	574	9.7	
Total 25 fam....	675	582	1257	46.3	Av. fam. 50.2	Total 22 fam..	1037	112	1149	9.7	Av. fam. 52.2

TABLE IV.

Starved. 1913–14. Parent winged.						*Control.* 1913–14. Parent winged.					
No. Exp.	No. Non-Winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.	No. Exp.	No. Non-winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.
Winged A^1 4....	65	2	67	.3	Starved 30 h.	1.....	15	0	15		
	—	–	—			3.....	26	0	26		
	65	2	67			5.....	25	4	29		
						7.....	28	0	28		
						9.....	27	0	27		
							—	–	—		
							121	4	125	3.	Av. family 25.
1914–15.											
Winged G (5).	29	0	29	0	♀ died young.	No.					
(6).	15	0	15		Starved 45 h.	controls					
	—		—								
	44		44								
Non-winged—											
G (2).....	11	8									
(8).....	44	16			" "	"					
(9).....	14	15									
	—	—									
	69	29	98	29.							

removed from the plants as soon as it was possible to determine the presence or absence of wing buds.

It has been suggested that young produced in the early part of the reproductive life of the mother would be better nourished and fewer wings would develop than among those born at the end of the period of reproduction. A consideration of the A1 and A2 series of Table I. seems to disprove this, as more wings developed in the families of the older members.

It might also be suggested that the effect of starvation was to reduce the size of the families and consequently the percentage of winged forms might be raised. In some cases the size of families of the starved mothers was smaller than that of the control, in other cases it was greater. The average sized family was 50.2 in the starved lines and 52.2 in the controls. The difference is not large enough to be important.

A few experiments were carried on with starved winged mothers. These are difficult to obtain, since the number normally produced is low and since it is impossible to distinguish wing buds at the early period when starvation is begun, thus the chances for finding winged forms among the starved young are slight. The control experiments (Table IV.), show that of the young of five winged mothers only 3 per cent. were winged. In the few families of starved winged mothers the percentage is less, often none of the young develop wings In passing it might be noted that in the G series (Table IV.) no. 5 and no. 6 winged individuals starved 45 hours produced no winged young, while their wingless sisters nos. 2, 8, 9, produced 29 per cent. winged forms. Apparently starvation has little effect on winged mothers. Teleologically this result would be expected. There would be no need for more winged forms in nature, since the parent had migrated to a fresh food supply.

In conclusion it seems most probable that the lessening of the food supply is the primary factor in determining the development of wings in the offspring of wingless mothers. The wing anlage appears to be present in all the parthenogenetic females and depends directly upon the condition of the food supply of the mother for its stimulation or suppression of development.

COLUMBIA UNIVERSITY,
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